ENZYME SYSTEMS

IN

MARINE ALGAE

-by-

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Thesis

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ABSTRACT OF THESIS

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Title of Thesis Enzyme systems in marine algae.

Some enzyme systems in extracts of the green alga Cladophora rupestris, the red alga Rhodymenia palmata and the brown algae Fucus vesiculosus and Laminaria cloustoni have been examined.

The α-amylase activity of C. rupestris was unaffected by added borate ions in contrast to results obtained by previous workers. Chloride, bromide and iodide ions exerted an activating effect on the amylase activity, decreasing in the order quoted, while fluoride ions inhibited the activity. The amylase activity was unaffected by added calcium ions but ethylene diamine tetra-acetic acid caused substantial inhibition, suggesting that the amylase is dependent for optimum activity on a metallic ion, which, if the algal amylase resembles other α-amylases, will be calcium.

As a preliminary to the investigation of the xylanase activity in algal extracts, the fine structure of the xylan from R. palmata was examined. This xylan has a degree of polymerisation of the order of 100 and may be slightly branched. Previous workers have shown that the xylan contains both β, 1-4 and β, 1-3 linkages. The present work has, by the 'Smith degradation'procedure and by enzymic degradation, shown that the β, 1-3 linkages are, in the main, singly placed in the xylan chain, with only a few placed adjacent to each other. The enzymic degradation was effected by a Myrothecium verrucaria preparation, since controls showed that extracts of C. rupestris and R. palmata contained trans-β-xylosylase activity synthesising β, 1-3 linkages. The presence of this activity, coupled with the very weak xylanase made the investigation of the latter activity impracticable.

The β-glycosidase activities of C. rupestris, R. palmata and L. cloustoni have been examined and compared with those of sweet almond emulsin. Using p-nitrophenyl-β-D-glycosides as substrates, the effect of fractionation, pH, temperature and aldono-lactone inhibition on the β-glucosidase, β-galactosidase and β-xylosidase activities of the algal extracts was examined. The β-glycosidase activities in the algal extracts were shown to be due to distinct enzymes in contrast to those of almond

emulsin/

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emulsin which cannot be separated by these techniques.

In investigating the initial stages of hexose metabolism in extracts of the four algae, a variety of weak phosphomonoesterase activities have been demonstrated. The most unusual, and in the case of the C. rupestris extracts, the strongest of these activities had optimum activity at pH 9·8 and was activiated by magnesium ions. Glucose-6-phosphate was a better substrate than β-glycerophosphate for the C. rupestris phosphatase between pH 6·2 and 7·7. The brown algal extracts did not contain any specific mannitol-1-phosphatase activity.

Phosphoglucose isomerase was demonstrated in C. rupestris and R.palmata extracts. C. rupestris extracts contained a very weak phosphoglucomutase. F. vesiculosus extract inhibited the phosphoglucose isomerase activity of almond emulsin.

ACKNOWLEDGEMENTS

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GENERAL INTRODUCTION

The marine algae make up the flora of the sea. Although a large number of microscopic species form part of the benthos and plankton, the larger species, evident on and close to the shores of the sea are the more obvious source of biochemical and commercial interest.

The algae generally, are divided into a number of classes. Of the more common of these classes, the blue-green algae (Myxophyceae or Cyanophyceae) are the most primitive, with little or no morphological development from the basic cellular structure. They have the ability, by virtue of this primitiveness, to live under conditions adverse to the growth of most other organisms. The green algae, (Chlorophyceae) form the most widely spread class, comprising unicellular and filamentous structures occuring as marine, fresh water and terrestrial forms. Botanically this class bears the closest relationship of the algae to higher plants. The red algae (Rhodophyceae) are split into two sub-divisions, Bangiales and Florideae. The former, minor division includes a variety of marine, fresh water and terrestrial forms, more primitive than their counterparts in the Chlorophyceae. The latter, major division covers mainly marine species which show a greater degree of specialisation with regard to their habitat than do the marine Chlorophyceae. The largest and most specialised algae are the brown algae (Phaeophyceae) which are almost exclusively marine. Morphologically and anatomically their structure is completely different to that of the higher terrestrial plants. A complete analysis of the structure and classification of the algae is given by Fritsch (1935, 1945).

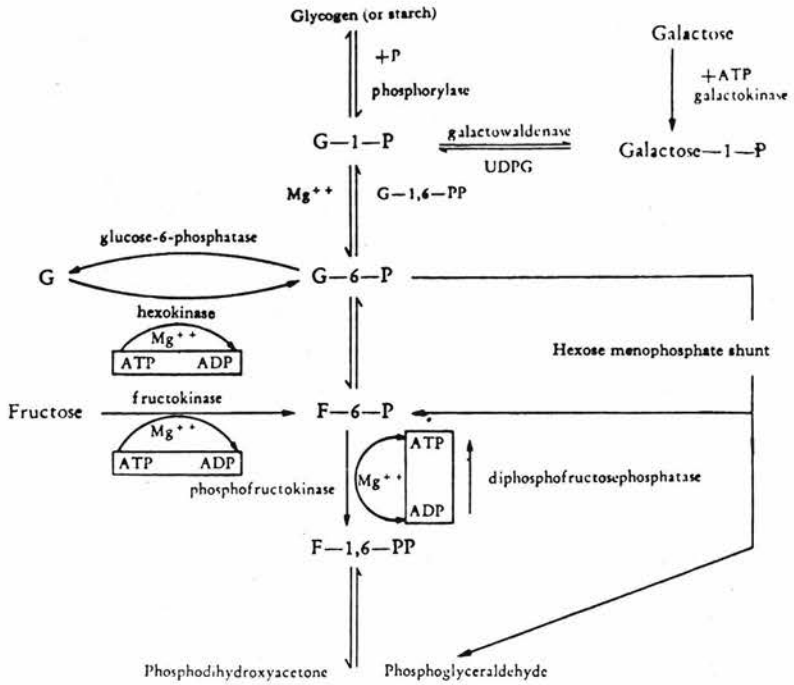


FIG. 1 —Preludes to glycolysis.

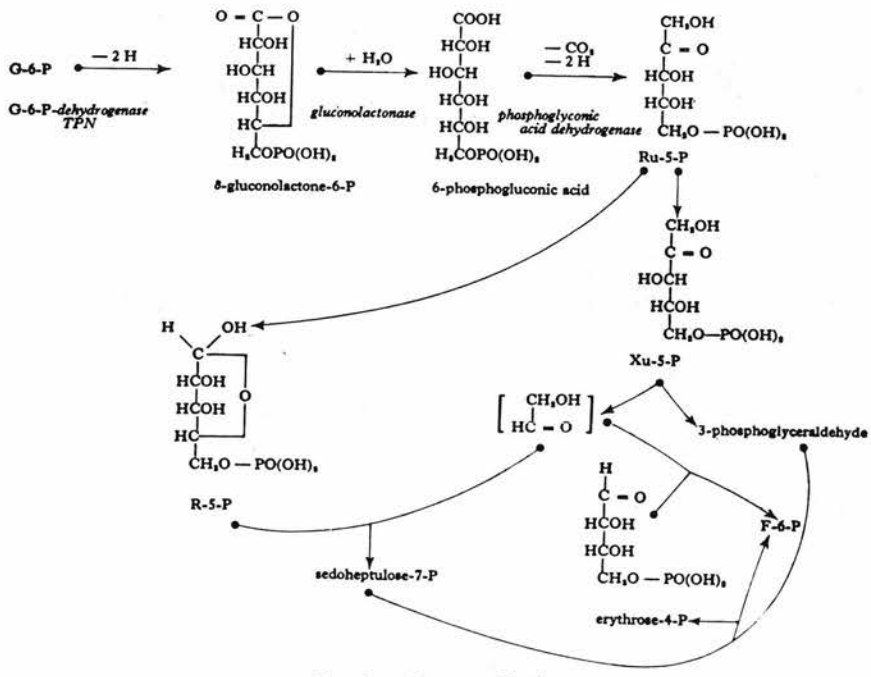


FIG. 2 —Pentose Cycle

Xu = xylulose, the ketose corresponding to xylose.

The growth and metabolism of algae has been reviewed by Fogg (1953). Algal enzyme systems, generally, have not been extensively examined. In the initial metabolism of carbohydrates dealt with here the accepted pattern of synthesis and degradation in higher plants and animals is outlined in Figs. 1 - 3. Acknowledgement is due here to Professor Marcel Florkin for his permission to reproduce these figures (Florkin, 1960). The role of uridine diphosphate hexoses and pentoses in the synthesis of polysaccharides has been reviewed by Leloir (1961).

Attempts to demonstrate the pathways outlined in algae have been mainly confined to the fresh water unicellular green algae, especially Chlorella sp., because of the relative ease of controlled culture experiments. The presence of a starch-type polysaccharide in Chlorella vulgaris was reported by Bailey and Neish (1954) and these workers also reported the presence of a phosphorylase and a branching enzyme similar to those in potatoes which catalysed the in vitro synthesis of an amylopectin/glycogen-type polysaccharide from glucose-1-phosphate. Holzer and Holzer (1952), in an extensive survey of the enzyme systems of Chlorella pyrenoidosa, failed to obtain a polysaccharide staining with iodine on incubation of extracts with glucose-1-phosphate. They did however demonstrate the expected pathway from glucose via hexose monophosphates, fructose, diphosphate, triose phosphates to phosphoglyceric acid. The enzyme catalysing the final step was shown to have properties similar to the triose phosphate dehydrogenase present in higher plants and animals. These workers therefore concluded that the carbohydrates in Chlorella were metabolised according to essentially the standard pattern. A minor variation has been reported by Warburg et al (1957) who obtained

D-(-)-lactic acid, rather than the expected L-(+)-lactic acid on incubating a Chlorella extract with pyruvic acid. Richter (1959, 1961) used Chlorella pyrenoidosa extracts as controls in a comparison with the enzyme systems of the unicellular blue-green alga Anacystis nidulans. The most striking result was the absence of fructose diphosphate aldolase in the Anacystis. In demonstrating enzymes of the pentose cycle, which are more active in Anacystis than in Chlorella, Richter suggests that the only pathway of hexose degradation in Anacystis is through the pentose cycle which also exists as an alternative pathway in Chlorella.

The presence and synthesis of a starch-type polysaccharide in another blue-green alga, Oscillatoria princeps, has been extensively investigated (Fredrick, 1962). Phosphorylase and branching enzyme have been demonstrated in varying ratio depending on the strain of alga examined, and it is postulated that this variation is responsible in turn for the variation from slightly branched amylose structures to highly branched amylopectin structures in the different strains.

The more widely documented carbohydrate content of marine algae may be used as an indication of the expected enzyme systems. Starch-type polysaccharides have been found in the green algae, Acrosyphonia, (O'Donnell and Percival, 1959), Caulerpa (Mackie and Percival, 1960), Enteromorpha, (McKinnell and Percival, 1962) and Cladophora (Love et al 1963). Meeuse and Kreger, (1959) examined a large number of iodine-staining, starch-type polysaccharides, from fresh water and marine algae, by means of X-ray diffraction studies. They showed a variety of patterns two of which were similar to those obtained from

cereal starch and from tuber starch. Amylase activity, using soluble starch as substrate, has been reported in extracts of the green algae Cladophora rupestris and Ulva lactuca, the red alga Rhodymenia palmata and the brown alga Laminaria digitata by Duncan et al (1956) who also reported that in the case of Cladophora rupestris extracts the mechanism of hydrolysis of starch and maltose, and other polysaccharides examined - Rhodymenia palmata xylan and laminarin - did not involve phosphorolysis. Duncan (1956) showed that Cladophora rupestris extracts also contained α-amylase activity. Hydrolysis of amylose, amylopectin and glycogen by an extract of the red alga, Porphyra umbilicalis was reported by Peat and Rees (1961). Both the Edinburgh and Bangor groups also reported substantial hydrolytic activity in their algal extracts towards floridean starch, a reserve amylopectin/glycogen-type polysaccharide, isolated from red algae by Fleming et al (1956) and by Peat et al (1959) from Dilsea edulis, and by Turvey and Rees (1958), from Porphyra umbilicalis. As an example of a more unusual enzymic activity, Peat and Rees (1961) showed that their extracts of Porphyra umbilicalis exhibited marked sulphatase activity towards the sulphate ester groups of the polysaccharide porphyran, which is characteristic of Porphyra sp. and contains D- and L-galactose, 3:6 anhydro-L-galactose, 6-O-methyl-D-galactose and galactose ester sulphate units (Turvey and Rees, 1958; Munn and von Holdt, 1957).

The low molecular weight carbohydrates of marine algae (Lindberg, 1956) present a more obvious picture of the differences between algae and higher plants. In a survey of photosynthetic products using labelled carbon dioxide, Bidwell (1958) showed that only the green algae

produced sucrose, the intermediate storage product of photosynthesis in higher plants, while the brown algae produced mannitol and the red algae produced floridoside (2-O-α-D galactosyl glycerol). Peat and Rees (1961) reported the presence of enzymes capable of hydrolysing sucrose and floridoside and Duncan et al (1956) demonstrated invertase activity in their extracts. The function of mannitol in the brown alga Fucus vesiculosus was examined by Bidwell and Ghosh (1962) using labelled mannitol. They showed that only a small amount of the added mannitol was metabolised and only part of the activity was extractable in polysaccharide soluble in dilute acid. It may be significant here to note the presence of small amounts of mannitol as an integral part of the structure of laminarin extracted from the brown alga Laminaria cloustoni (Arman et al 1962) although no enzymes involved in the metabolism of mannitol in algae have been demonstrated apart from the circumstantial evidence provided by radio active tracer work.

The mode of enzymic synthesis of marine algal carbohydrates is still largely unknown. As mentioned previously, the degradation of the polysaccharides, starch, laminarin and xylan examined by Duncan et al did not take place by phosphorolysis and hence, the reverse reaction presumably would not occur. Using an extract of Cladophora rupestris, Duncan (1956) was unable to demonstrate any net gain in polysaccharide staining with iodine on incubation with soluble starch as primer and excess glucose-1-phosphate. In connection with this part of glucose metabolism it is of interest to note that Jacobi (1957a) was unable to show phosphoglucomutase activity in the green alga Ulva lactuca. Duncan and Manners (1958) reported the presence of trans-α-glucosylase

activity in Cladophora rupestris and Duncan et al (1959) demonstrated trans-β-glucosylation in the green algae Cladophora rupestris and Ulva lactuca. It is however unlikely that these enzymes alone are responsible for polysaccharide synthesis in the algae mentioned. Bean and Hassid (1955) in photosynthesis experiments with labelled carbon dioxide demonstrated the presence of uridine diphosphate-glucose and-galactose in the red alga Iridophycus flaccidum and proposed a mechanism involving these nucleotides in the synthesis of floridoside and other more complex polygalactose structures in this alga. More recently, Hassid and Su (1962) have reported the identification of uridine diphosphate-D-glucose and uridine diphosphate-D-galactose, together with uridine diphosphate glucuronic acid, guanosine diphosphate-D-mannose and guanosine diphosphate-L-galactose in an ethanolic extract of the red alga Porphyra perforata. Based on these results they present a hypothesis for the synthesis of the complex polysaccharide, pophyran, mentioned above, involving the guanosine diphosphate sugars in the insertion of the L-galactose residues and the uridine diphosphate-D-glucose and -D-galactose in the insertion of the D-galactose residues. It is not improbable that the synthesis of polysaccharides in the algae as a whole will be shown to follow the same pattern involving these nucleotide compounds as has been discovered in recent years in higher plants and animals (Leloir, 1961).

The eventual breakdown of glucose and its entry into the metabolic cycles in marine algae shows a number of marked deviations from the standard pattern. Watanabe (1932, 1937) showed that whereas the addition of glucose to a culture of Chlorella sp. stimulated increased

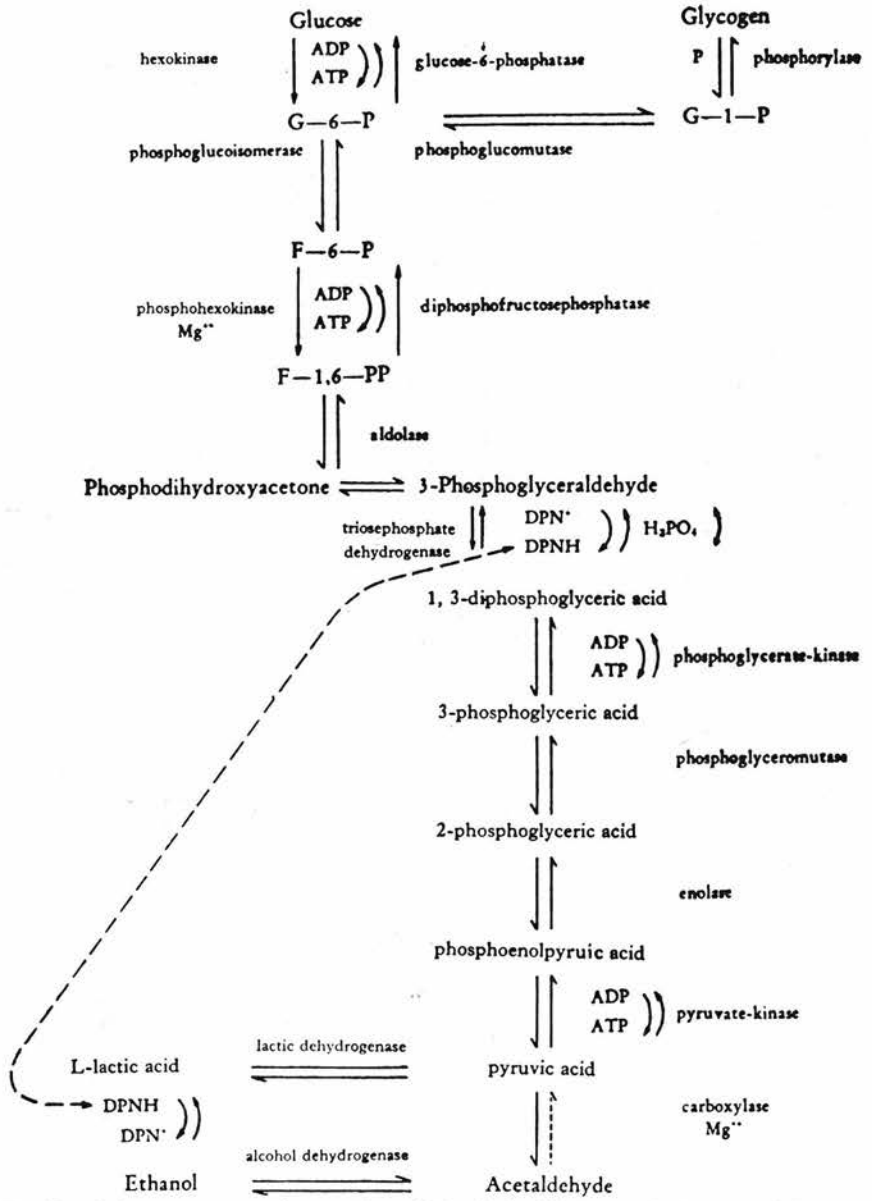


FIG. 3 —Scheme to summarize reactions of glycolysis. The entry of glucose and glycogen is shown. For other entries see Fig. 1 Two terminations are shown : that of alcoholic fermentation and that of lactic fermentation.

metabolic activity, the addition of glucose to metabolising marine algae had little or no effect. He did not discount the possibility of permeability factors preventing the entry of the glucose into the algae but his results were confirmed by Jacobi (1957a and b) who was unable to demonstrate hexokinase in the green algae Ulva lactuca, Chaetomorpha linum, and Bryopsis plumosa and in the red algae Delisseria sanguina, Phycodrys rubens, Polysyphonia violacea and Cystoclonium purpurescens. Bean and Hassid (1956) showed that extracts of the red alga Iridophycus flaccidum were able to oxidise glucose and galactose directly to gluconic and galactonic acid respectively. Bidwell and Ghosh (1963) have shown that the brown alga, Fucus vesiculosus was able to metabolise labelled glucose to a small extent. Radio activity was detected in extracted polysaccharides and sugar phosphates but none was found in mannitol or phosphoglyceric acid. The absence of activity in the phosphoglyceric acid is noteworthy in that phosphoglyceric acid forms part of the glycolytic pathway of glucose breakdown shown in Fig. 3. Labelled phosphoglyceric acid was demonstrated as one of the primary products of photosynthesis of labelled carbon dioxide by the red alga Iridophycus flaccidum (Bean and Hassid, 1955) but whether it was taking part in glucose metabolism was not shown. Jacobi (1957a and b) demonstrated fructose diphosphate aldolase, glucose-6-phosphate dehydrogenase and triose phosphate dehydrogenase only in the three green algae examined above for hexokinase. He was unable to demonstrate any of these activities in the four red algae surveyed. He concluded that a mechanism replacing the glycolytic cycle must be present in the red algae. This is consistent with the glucose and galactose oxidase

activities reported by Bean and Hassid. Another variation from the standard scheme (Fig. 3) was the absence of lactic dehydrogenase and alcohol dehydrogenase in all the green and red algae examined.

It is of interest to note that although the first step in the hexose monophosphate shunt was not demonstrable in red algae, that is, the ~~reduction~~ oxidation of glucose-6-phosphate by glucose-6-phosphate dehydrogenase, Bean and Hassid (1955) found sedoheptulose-7-phosphate in extracts of Iridophycus during their photosynthesis experiments. This prompts the suggestion that by means of the glucose oxidase mentioned previously, a direct entry into the pentose cycle could be achieved if the gluconic acid were then phosphorylated. Another possibility is that the carbohydrate metabolism of the red algae could be based on galactose rather than glucose in view of the predominance of this sugar in the carbohydrates of these algae.

It is apparent that a great many problems remain to be solved in relation to the initial carbohydrate metabolism of the marine algae. The green algae make the closest approximation to the pathways demonstrable in higher plants and animals but there is no evidence for the presence of these pathways as a significant factor in the metabolism of red algae. On the contrary it appears that a completely new scheme must be devised. No specifically enzymic examination of the metabolism of the brown algae seems to have been made.

The present work was undertaken in order to investigate various aspects of the initial metabolism of carbohydrate material in a number of marine algae. The α-amylase present in Cladophora rupestris was examined to compare its properties with those of other amylases in view

of the reported presence of a starch type-polysaccharide in this alga. The fine structure of Rhodymenia palmata xylan has been examined with a view to investigating the enzymic degradation of this xylan by algal extracts. A study of the β-glycosidase activity of Cladophora rupestris, Rhodymenia palmata and Laminaria cloustoni and of the β-glycosidase activity of a sweet almond (Prunus amygdalus) preparation has been undertaken in order to compare the range of specificity in the algal preparations with that in higher plants as typified by the almond preparation. In view of the reported deviations from the standard metabolic cycles noted in the foregoing pages the initial stages of hexose and hexose phosphate metabolism have been investigated using extracts from Cladophora rupestris, Rhodymenia palmata, Laminaria cloustoni and Fucus vesiculosus.

METHODS AND MATERIALS

Section I

Measurement of enzymic activity.

α-Amylase assay.

This method is based on the iodine staining procedure of Van Dyk and Caldwell (1956). Samples (2 ml.) from the assay digests containing soluble starch, final concentration 0.012 - 0.025%, and the enzyme preparation were added to 2 ml. of a solution of iodine in potassium iodide (0.2% in 2.0%) and the volume made up to 10 ml. The extinction (E) of this solution was determined using filter 626 (approx. 600 mμ) in an E.E.L. colorimeter and the extinction ratio (E.r.), the ratio of the

extinction after incubation time T to the extinction at zero time, calculated. The extinction ratios were plotted against time and the reciprocal of the time taken to reach a particular E.r., usually 0.5, used as a measure of the amylase activity.

The validity of the results obtained by this method were checked in digests containing 2.5, 5.0, 7.5 and 10.0 ml. of Cladophora rupestris extract C (600 mg./30 ml. water) with soluble starch and appropriate volumes of water to a digest volume of 20 ml. The activities, with T 0.5 measured in hours because of the inherently low activity of the extract, were 0.19, 0.39, 0.67 and 0.82 units respectively. These results give a linear plot passing through the origin, thus confirming the suitability of this method for this range of extract concentrations.

All amylase assay digests were incubated at pH 5.6 which was within the optimum range observed by Duncan (1956).

β-Glycosidase assay.

Hydrolytic activity towards p-nitrophenyl-β-D-glycosides was measured by determining the p-nitro-phenol liberated as the more strongly coloured nitro-phenate ion which exists in alkaline solution. Samples (1 ml.) were taken from digests of suitable substrate concentration, added to B.D.H. Universal buffer solution pH 9.8 (5 ml.) and the extinction of the resulting solution measured at 400 mμ. This is a modification of the method used by Woolen et al (1961).

Other enzymic assays were carried out by estimating the products or disappearance of substrate according to one of the methods outlined below.

Analytical methods.

Reducing sugars were estimated by the method described by Somogyi (1952). The method of Park and Johnson (1949) was used to determine micro-quantities of sugars. The following standard procedure was adopted. Digest solutions containing substrate (2 ml. 0.001M), enzyme solution (2 ml.), buffer (1 ml.) and water (1 ml.) were incubated and then diluted to 10 ml. by the addition of zinc sulphate (2 ml. 5%) and barium hydroxide (2 ml. saturated solution) solutions for deproteinisation (Nelson, 1944). A sample of the deproteinised solution, (1 ml.) was diluted with 4 ml. water and 1 ml. of the resulting solution added to 1 ml. potassium ferricyanide solution (0.5 g/litre) and 1 ml. sodium carbonate/potassium cyanide solution (5.3 g., 0.65 g/litre) and heated on a boiling water-bath for 20 minutes. After cooling, 5 ml. of a ferric iron solution (1.5 g. ferric ammonium sulphate; 1 g. Duponol (sodium monolauryl sulphate) /litre) were added and the colour developed for 15 minutes before reading the extinction at 690 mμ. Commercial Duponol was recrystallised five times from ethanol and the final ferric iron solution filtered before use. This procedure is such that 2 ml. 0.001M substrate in a digest of 6 ml. gave a sample within the limits of the method, that is, between 1-9 μg of monosaccharide.

The phenol/sulphuric acid method of Dubois et al (1956) was used in the detection and estimation of micro-gram quantities of reducing monosaccharides and oligosaccharides containing reducing sugar residues.

This method was also used to determine the degree of polymerisation of small oligosaccharides (Timell, 1960). The apparent reducing monosaccharide content was determined by the phenol/sulphuric acid method before and after reduction of the oligosaccharide by potassium

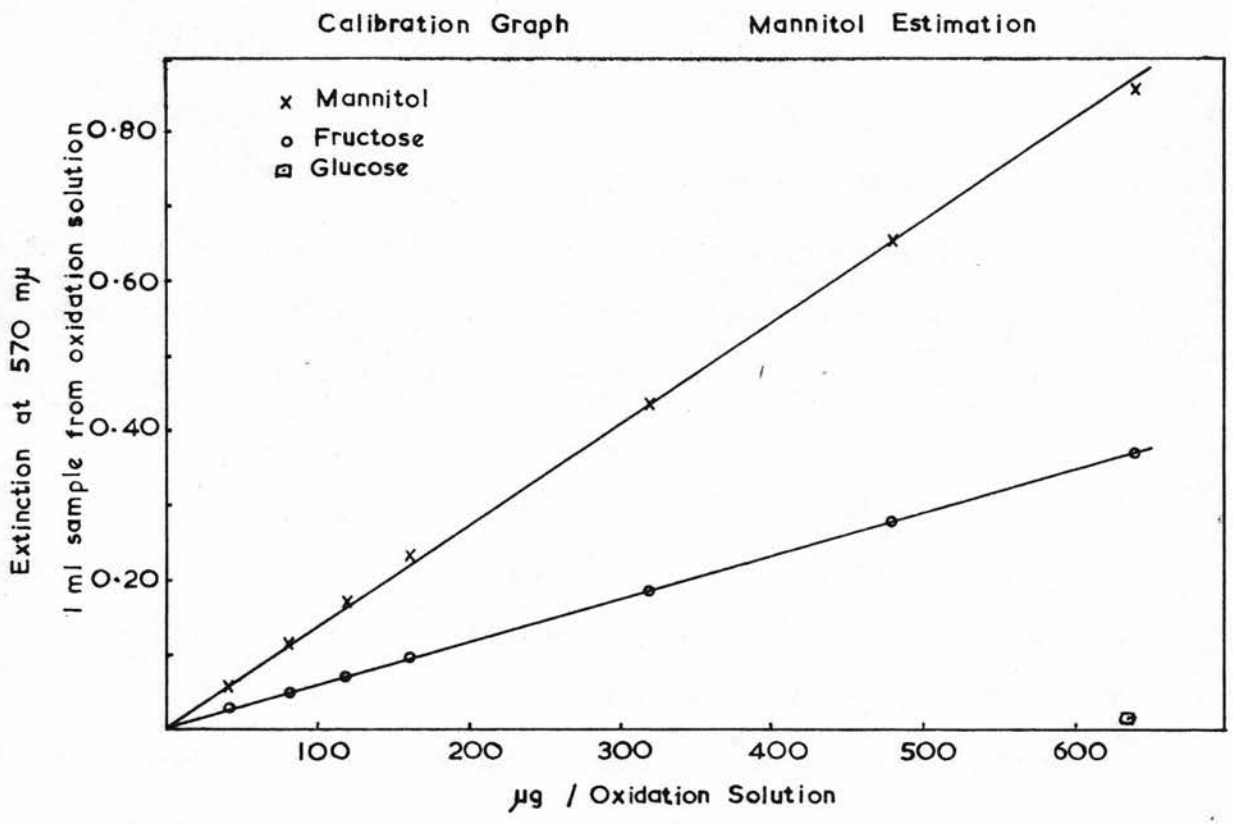


FIGURE 4

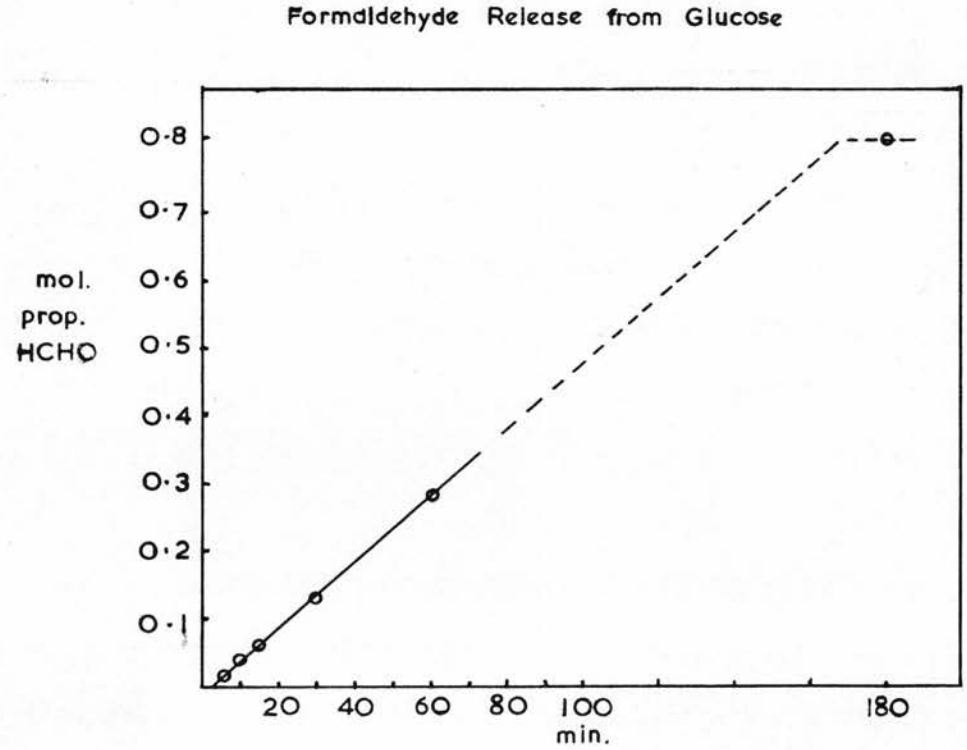


FIGURE 5

borohydride. The ratio of these two results was compared with a plot of the theoretical ratios for di-, tri- and higher oligosaccharides to give a value for the apparent D.P.

Fructose and fructose-6-phosphate were estimated by the method of Roe et al (1949) described by Ashwell (1957). Samples containing up to 500 µg. of fructose or fructose-6-phosphate were used but the intensity of the colour was very dependent on the conditions of development, making it necessary to include known standard solutions with any absolute concentration measurements.

Mannitol and mannitol-1-phosphate were estimated by an adaptation of the method described by Burton (1957). Up to 500 µg. hexitol or hexitol phosphate in 4 ml. water were oxidised with 2 ml. 0.1M periodic acid for 5 min. in the presence of 1 ml. of 2.5N sulphuric acid at room temperature. The oxidation was stopped by the addition of 3 ml. M. sodium sulphite and the formaldehyde content of a 1 ml. sample determined by the chromotropic acid method. (Section III). Calibrations for mannitol and fructose are shown in Fig. 4. Liberation of formaldehyde from glucose was negligible after 5 min. oxidation. The results shown in Fig. 5 were obtained in an experiment to determine the rate of formaldehyde release from glucose oxidation. Glucose yields 0.8 molar proportions of formaldehyde after less than three hours. Fructose yields 0.8 mol. prop. after 5 min. and mannitol 1.9 mol. prop. also after 5 min. oxidation.

Inorganic phosphate was determined by the method of Allen (1940) modified by Liddle (1956) on a reduced scale. The original method used 60% perchloric acid, 10% ammonium molybdate in 5% ammonium hydroxide (S.G. 0.88)

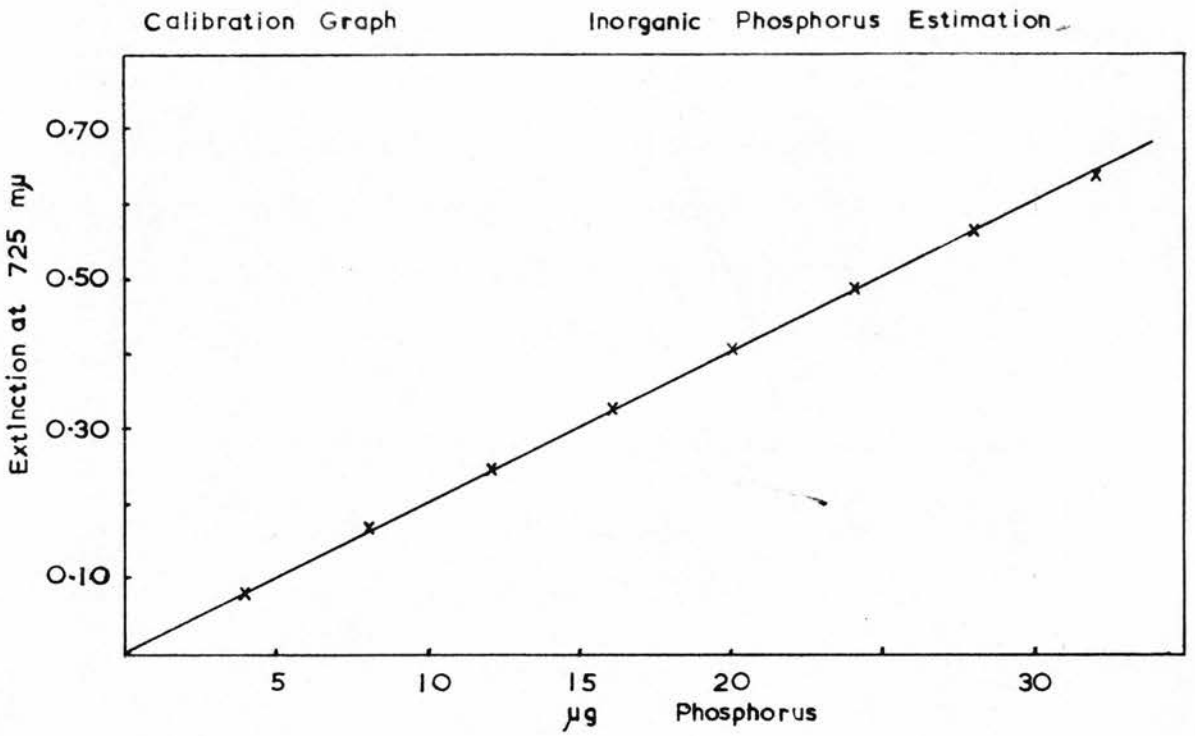


FIGURE 6

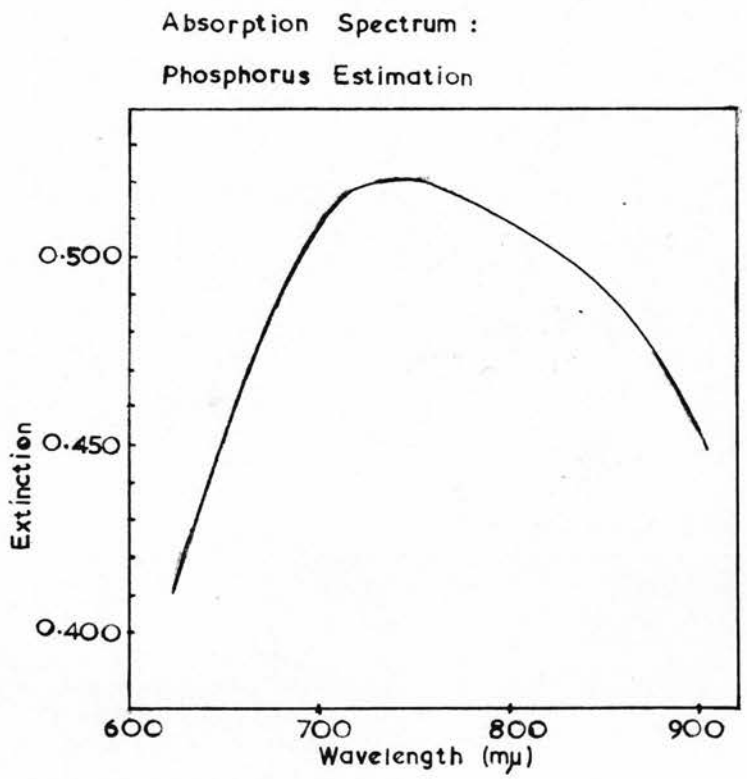


FIGURE 7

and an Amidol (2:4 diamino phenol-hydrochloride) solution containing 1% Amidol in 20% sodium bisulphite. These reagent solutions were prepared and diluted as required, the perchloric acid and Amidol, 2.5-fold and the ammonium molybdate 5-fold. The digest solution (2 ml.) containing less than 40 µg. inorganic phosphorus was added to 1 ml. of the dilute perchloric acid reagent and the dilute Amidol and molybdate reagents added in that order. The extinction of the resulting solution was read at 725 mµ after 15 min. development. The calibration graph is shown in Fig. 6. The extinction-wavelength curve is shown in Fig. 7.

General.

All extract solutions and digests were maintained under a layer of toluene to prevent the growth of bacteria and moulds.

The buffer systems used are listed by Gomori (1955); B.D.H. Universal buffer was also used.

pH measurements were made on a Pye Universal pH meter.

Spectrophotometric determinations were done on either a Unicam S.P. 500 or 600 spectrophotometer.

Section II

Chromatography

Paper chromatography

The solvent systems used are listed below. In the text they are referred to in the abbreviated ratio form indicated.

6/4/3: n-butanol/pyridine/water.

10/3/3: n-butanol/pyridine/water.

10/4/3: ethyl acetate/pyridine/water.

9/1/1: methyl ethyl ketone/acetic acid/water, saturated with boric acid.

mek/water: methyl ethyl ketone saturated with water.

200/17/1: methyl ethyl ketone/water/ammonium hydroxide (S.G. 0.88).

The spray reagents used were the aniline hydrogen phthalate and oxalate of Partridge (1949) for reducing sugars, and the alkaline silver nitrate spray reported by Trevelyan et al (1950) for non-reducing sugars. The sodium meta-periodate/potassium permanganate spray developed by Lemieux and Bauer (1954) was also used.

Electrophoresis.

The Shandon horizontal electrophoresis apparatus used was kindly loaned by Dr. H.T. McPherson. Electrophoreses in 0.1 M borate buffer pH 10.0 at 350 v., 5-12 ma., were run for 30 - 60 minutes on Whatman No. 1 paper cut to 22 x 7.5 cm. Tetra O-methyl glucose was used as a 'non-mobile' marker. The spray reagents listed above were used. The aniline phthalate spray was necessary to reveal the tetra O-methyl glucose and as a general rule when using this spray two treatments and heating at 130-140°C. gave the best results.

The position of sugars on chromatograms is related to the solvent front or the position of xylose by calculating the ratio of the distance moved by the spot to that by the solvent front (R_f) or by xylose (R_x). On electrophoretograms the ratio of the distance moved to that moved by xylose from the zero, marked by the distance moved by tetra O-methyl glucose is quoted as the M_x value.

Column chromatography.

Charcoal/Celite columns were prepared and eluted according to the

procedure described by Whistler and Durso (1950). Charcoal pads (Andrews et al, 1956) were also employed in the separation of oligosaccharides.

Sephadex columns were prepared as described by Flodin (1962).

Di-ethyl amino ethyl (DEAE) cellulose (Petersen and Sobers, 1956) was used in an attempt to purify the amylase in Cladophora rupestris extracts. Using the method described by Pasur and Ando (1959) columns were set up at a temperature of 2°C. The rate of elution of the column after adsorption of the crude algal extract became so slow that this method was impracticable under the conditions used.

Using DEAE cellulose substantial amounts of the inert protein can be removed from crude algal extracts as was shown by the experiments described below.

Cladophora rupestris extract C (1.5 g.) was dissolved in McIlvaine buffer (180 ml. pH 7.2). The centrifugate was divided into three 60 ml. aliquots and the pH of two of these adjusted to 4.2 and 6.0. Each of the three solutions was divided in two, and 10 g. DEAE cellulose (OH' form) added to one solution of each pH. All solutions were kept at 2°C. for 30 min. and the DEAE cellulose filtered off. As a measure of the amount of protein material removed by the DEAE treatment the extinction of the treated and untreated solutions were determined at 282 mμ. Results showed that in all cases approximately 90% of material absorbing at this wavelength had been removed.

The pH of filtrates and untreated solutions were adjusted to pH 5.8 and digests, prepared with 4 ml. extract solution and 1 ml. carbohydrate solution (2 mg./ml.) were incubated for 7 days at 35°C. Laminarinase,

amylase and maltase activities were determined by means of these digests. Activities were assayed by measurement of the reducing powers of the whole digests by the Somogyi method.

Assuming the untreated solutions to have 100% activity the percentage activity not adsorbed by the DEAE cellulose treatment was

pH 4.2	Laminarinase 45%	Amylase 35%	Maltase 15%
6.0	70%	35%	20%
7.2	40%	30%	20%

Treatment at pH 6.0 is the most effective from the point of view of unadsorbed activity although dilutions caused by pH adjustment are not taken into account. The untreated solutions were kept as far as possible under the same conditions as the treated solutions in order that losses of activity due to deactivation should be comparable.

Similar experiments were carried out in which extract solutions were filtered through a pad of DEAE cellulose under suction. Both OH^{-} and Cl^{-} forms of the cellulose were used, extinction measurements as previously described showed approximately 80% removal of material absorbing at 282 mμ in both cases.

An examination of the effect of this method was carried out at pH 6.0 and digests prepared without pH adjustment. Percentage activities of the filtrate were laminarinase 85%, amylase 80%, maltase 65%, xylanase 85%.

These results indicate that this method should be of value in providing for the speedy removal of inert protein material in algal extracts.

Section III

Periodate oxidation

The reduction of periodate was followed by the spectrophotometric method of Aspinall and Ferrier (1957) in which the extinction of very dilute periodate solutions was measured at 222.5 mμ.

Formaldehyde released during oxidations was determined by the chromotropic acid method of Frisell et al (1954) as adapted by Cunningham (1961).

Formic acid was determined by titration with dilute barium hydroxide using a Pye Universal pH meter. The end-point was found by drawing pH/titre curves and reading off the titre at pH 5.8. This was found to be more reliable than direct titration to pH 5.8. The barium hydroxide solutions were standardised against benzoic acid using the same technique with an end point of pH 7.0. All titrations were done under an atmosphere of nitrogen to prevent precipitation of barium carbonate.

Section IV

Methylation

Methylations of oligosaccharide material were carried out according to the procedure developed by Kuhn et al (1955) from the original method of Purdie and Irvine (1903). Oligosaccharide (0.5-2.0 mg.) was treated with methyl iodide (0.2 ml.), di-methyl formamide (0.2 ml.) and silver oxide (0.2 g.). The methylation mixtures were mixed in the dark on electrically driven rollers in preference to stirring or shaking because of the small scale of the operations. Virtually complete methylation,

as observed by paper chromatography of the hydrolysed product, was obtained after two 18-24 hr. treatments.

The ratio of spots developed on paper chromatograms, after acid hydrolysis of methylated oligosaccharides was determined by the procedure described by Wilson (1959). The hydrolysate was chromatographed in 200/17/1 and sprayed with the aniline phthalate spray. The spots were eluted with 4 ml. 0.7 N. hydrochloric acid in 80% ethanol, by contact for 1 hr. and the extinctions of the resulting filtered solutions measured at 360 mμ. Paper controls for the background colour on the chromatogram were determined simultaneously.

Section V

Enzyme preparations

Cladophora rupestris

Fresh, washed and sorted seaweed (2 kg.) collected near North Berwick in October 1960 was minced and extracted with 0.25% sodium carbonate (Duncan et al, 1956). The protein was precipitated from the dialysed extract with ammonium sulphate (0.7 saturation), dissolved in water and dialysed. After dialysis for 18 hr. a green precipitate was formed, leaving a brownish supernatant solution. These two apparent fractions were separated and the dialysis continued.

After two days dialysis half the brown solution was freeze-dried giving fraction I (3.7 g.). The green precipitate was freeze-dried after three days to give fraction II (16.2 g.), and after four days the remainder of the brown solution was freeze-dried to give fraction III (1.5 g.).

Fraction II (8 g.) was extracted by the same procedure giving fraction IIa (6 g.) and fraction IIb (0.5 g.).

A further extract, prepared in October 1958 by Dr. W.L. Cunningham is referred to in the ensuing text as extract C.

The following experiments, pertinent to the collection and storing and to smallscale extraction procedures were carried out.

Frozen seaweed (20 g.) was ground with sand (100 g.) in a mortar for 5 min. and extracted with two 30 ml. portions of B.D.H. buffer pH 5.6 for 15 min. The temperature of the extraction never exceeded 0°C. Both pale green extract solutions were filtered through DEAE cellulose and their amylase activity measured against soluble starch (Sections I and II above). A solution of extract C was used as a control.

Activities expressed as 1/T0.5 hr. were: extract C, 0.43 units; first extract 0.22 units; second extract 0.22 units. These figures indicate that the extraction was not complete. Rough measurements by evaporation and weighing, showed that the extracts contained approximately the same weight of dissolved material as extract C.

A second extraction was carried out using broken glass instead of sand to effect disintigration of the algal cells. The ground seaweed was then extracted with 50 ml. 0.2M acetate buffer pH 5.6 for 3 hr. with occasional grinding and mixing. The dark green extract was filtered through DEAE cellulose and the amylase activity determined as 0.32 units. The strong green colour of this extract suggests a more efficient extraction but no marked improvement has been made in the amylase activity.

Fresh seaweed (100 g.) was minced and ground with broken glass (100 g.) at intervals for one hour, kept at -15°C. for 18 hr. ground to a powder while still frozen and extracted with acetate buffer (pH 5.6, 60 ml.) for two hours. The amylase activity was found to be 1.5 units. The intense

green colour of this extract solution was only partially removed by the DEAE cellulose treatment and subsequent qualitative experiments showed that excessive quantities of DEAE cellulose would be required to effect complete removal of the colouring material. Thus the use of this method is impracticable in dealing with efficient initial extracts, which compares with its successful application to the purification of extract C solutions described in Section II.

The frozen seaweed on which the initial experiments were carried out had been frozen immediately after collection and prior to final washing and sorting. It is suggested therefore that on thawing this seaweed, cell rupture took place and substantial amounts of the activity was leached during the washing and sorting. On the other hand, the second batch of seaweed had been thoroughly sorted on collection and was extracted without any further treatment, resulting in a markedly higher amylase activity.

Rhodymenia palmata

The seaweed used was collected by Mr. E. Booth of the Institute of Seaweed Research in October 1961. Two extractions (2.5 kg. each) were carried out with 0.25% sodium carbonate and 0.05 M McIlvaine buffer pH 7.0 (4 litres of each). After dialysis, ammonium sulphate precipitation (0.8 saturation) and dialysis of the precipitate, freeze-drying yielded 4 g. of the carbonate extract (C) and 6 g. of the buffer extract (P). Both extracts showed fairly strong activity in digests with laminarin, maltose, p-nitrophenyl β-D-glucoside and -xyloside and weak activity towards soluble starch and xylan (from Rhodymenia palmata).

Laminaria cloustoni

Fresh seaweed, collected by Mr. Booth in December 1961 (5 kg.) was extracted with sodium carbonate. After dialysis, protein and a large quantity of alginate was precipitated from 0.8 saturated ammonium sulphate solution, suspended in water and dialysed for 5 hr. to remove most of the sulphate. The alginate in the solution was precipitated by adding calcium chloride until no further precipitation took place. Protein was precipitated from the solution with ammonium sulphate as before but after 6 hr. at 0°C. the solution had set into a gel. The insoluble material, assumed to be protein and calcium alginate, was suspended in water and dialysed. Material which remained insoluble was rejected as calcium alginate and the sulphate free solution freeze-dried to yield 20 g. of a white powder. The activity of this extract towards the substrates used with Rhodymenia extract above was very weak. No hydrolytic activity was observed in digests with maltose even after 5 days incubation.

Myrothecium verrucaria

Using a suspension of spores from a mature culture of the mould obtained from the Commonwealth Mycological Institute, Kew, Surrey, and the culture medium used by Whitaker (1953) a number of shake cultures were prepared and kept at 25°C. for 14 days. The culture was filtered through a sintered glass filter, the filtrate buffered to pH 6.0 and then freeze-dried. Bishop and Whitaker (1955) reported an optimum pH of 6.0 for xylanase activity in their Myrothecium preparation.

In view of the reported specificity of Myrothecium preparations for

the hydrolysis of β,1-4 linked xylose units (Bishop 1956) and its projected use in investigations of the fine structure of Rhodymenia palmata xylan (Chapter II) test digests with colloidal xylan solutions were prepared (Sørenson,1957). Substantial activity was observed chromatographically in digests with the β,1-4 linked esparto xylan, (Chanda et al 1950) but only a trace of xylose was detected after 14 days incubation with the β,1-3 linked xylan from Caulerpa filiformis (Mackie and Percival, 1959).

Sweet almonds

Defatted almond powder (200 g.) obtained by the procedure described by Cunningham, (1961) was extracted with ethanol (33%, 1600 ml.) for 10 min. The extract was filtered and the residue (150 g.) retained. The filtrate was cooled to 0°C. and 800 ml. 95% ethanol added. The precipitate was dissolved in water, dialysed against running tapwater and freeze-dried to yield 6 g. of almond protein preparation hereafter referred to as almond emulsin.

Section VI

Preparation of substrates

Rhodymenia palmata xylan

The residue from the protein extraction described in the previous section (3 kg.) was stirred for 2 days at room temperature with hydrochloric acid (2 N. 7 litres) (Barry and Dillon, 1940). After removal of insoluble material, the solution was made to 50% acetone, and the precipitate washed free of acid and dried with acetone, alcohol and

ether to yield 160 g. of a pale purple powder.

This crude product (30 g.) was dissolved in water (about 2 litres) at 70-80°C. by heating for about an hour. The dark purple residue was removed and the xylan reprecipitated from 50% acetone and dried to yield 20 g. white powder. A sample (5 g.) was dissolved in water and dialysed against running water for 3 days, and again reprecipitated from 50% acetone and dried to give a white powder (4 g.). Hydrolysis of this powder (xylan M) with sulphuric acid (2N. 98°C., 3 hr.) gave only xylose on chromatography. The xylose content determined by the Somogyi method was 99%.

Xylan D was prepared by Dr. W.A.M. Duncan following a similar procedure to that above with a final purification by means of the copper complex. Dr. Duncan also prepared the xylanitol sample used, by borohydride reduction of this sample of xylan, in which the terminal reducing xylose residue was reduced with potassium borohydride.

Xylan W kindly given by Dr. R.A. Wall, was prepared by extraction in n-butanol/water mixtures.

Xylobiose and xylotriose were obtained by the partial acid hydrolysis of the β,1-4 linked esparto xylan (Chanda et al 1950) according to the procedure described by Whistler and Tu (1951, 1952). Both products were obtained as crystalline hygroscopic solids and their specific rotations in water measured as -24.5° (xylobiose) and -43.7° (xylotriose) Whistler and Tu report -25.5° and -47° respectively.

In order to obtain standard β,1-3 linked xylo-oligosaccharides Caulerpa filiformis xylan (50 mg.) (Mackie and Percival, 1959) was hydrolysed at 98°C. in 5 ml. 0.2 N sulphuric acid. Samples were removed at intervals up to 60 min., neutralised, and chromatographed. All the

fractions contained oligosaccharides in decreasing amount, and were combined and concentrated to be used as chromatographic standards. Chromatographed in 10/3/3 and sprayed with aniline phthalate this mixture gave a series of spots with R_x values of 0.75, 0.41, 0.24 and 0.13. The first of these agrees with a figure of 0.77 reported by Howard (1957) for rhodymenabiose in this solvent system. Calculating the R_f values of these oligosaccharides from the R_f of xylose, 0.31, and plotting log. $(1/R_f-1)$ against the D.P. (Bate-Smith and Westall, 1950) a straight line relationship was obtained indicating that these oligosaccharides formed a homologous series. In passing it was noted that the 1-3 linked trisaccharide, rhodymenatriose, had the same mobility as xylobiose (0.41 and 0.39) and also the pentaose, rhodymenapentaose, had the same mobility as xylotriose (0.13 and 0.14). Similarly, rhodymenatetraose had the same mobility as the trisaccharide containing both β,1-3 and β,1-4 linked xylose residues isolated by Howard from an enzymic hydrolysis of Rhodymenia palmata xylan (0.24 and 0.25).

p-Nitrophenyl β-D-glycosides

The various glycosides were prepared by the methods indicated. De-acetylation was carried out by the procedure described by Leaback (1960) using barium methoxide in dry methanol.

p-Nitrophenyl β-D-xyloside: 1-bromo, 2, 3, 4, triacetyl α-D-xylose was prepared by the bromination of tetra-acetyl β-D-xylose (Hudson and Johnson, 1915) as a white crystalline solid mp. 99-101°C. $(\alpha)_D$ +211.4° (chloroform). Hudson and Johnson reported 101-102°C. and $(\alpha)_D$ +211.9°. This product was condensed with p-nitrophenol (Nath and Rydon, 1954). The de-acetylated xyloside had mp. 154-155°C. mixed mp. 154-156°C. with a sample provided by Dr. M.A. Jermyn. The specific rotation was

determined as -58.2° (in chloroform) Loontiens (1961) quoted -56.7°.

p-Nitrophenyl β -D-glucoside: this was prepared by the direct condensation of p-nitrophenol with penta-acetyl glucose (Jermyn, 1954). After recrystallisation and de-acetylation white needles mp. 164°C. were obtained. Montgomery et al (1942) quoted 164°C. for the mp. of p-nitrophenyl β -D-glucoside.

p-Nitrophenyl β -D-galactoside: aceto-bromo galactose was condensed at room temperature with sodium nitrophenate in aqueous acetone (Aizawa, 1939). The recrystallised, de-acetylated product had mp. 170°C. $(\alpha)_D$ -83.1° (chloroform) Aizawa quoted 170°C. and -74.3°; Snyder and Link (1953) quoted $(\alpha)_D$ -85°.

All the sugar phosphates used (barium and potassium salts) were commercial samples with the exception of mannitol-1-phosphate, the potassium salt of which was prepared by an adaptation of the procedure described by Wolff and Kaplan (1956). After reduction of mannose-6-phosphate (potassium salt) with potassium borohydride, excess borohydride was destroyed by adjusting the pH to 3.5 with dilute hydrochloric acid. Borate was removed by evaporation with methanol and the product made to standard volume and stored at -15°C. Acid hydrolysis, (2N. sulphuric acid, 6 hr. 98°C.) gave no products as examined by paper chromatography. This agrees with the acid stability reported for mannitol-1-phosphate by Wolff and Kaplan (1956). Estimation of apparent mannitol-1-phosphate by the mannitol estimation described in Section I gave an apparent 80% yield as compared with the initial mannose-6-phosphate content.

Barium salts of sugar phosphates were converted to the potassium salts by treatment with the theoretical amount of potassium sulphate in aqueous solution.

Chapter I

INVESTIGATION OF THE ∝-AMYLASE FROM CLADOPHORA RUPESTRIS

The hydrolysis of the ∝, 1-4 linkages in starch-type polysaccharides is catalysed by amylases. These are of two main types. ∝-Amylase, which catalyses the random hydrolysis of ∝, 1-4 linkages has been demonstrated throughout a wide range of animals and plants (Fischer and Stein, 1960). β-Amylase, on the other hand, which catalyses the stepwise degradation of the polysaccharide, with the release of maltose, occurs mainly in the higher plants (French, 1960).

The presence of amylase activity in Cladophora rupestris was reported by Duncan et al (1956). Duncan (1956) showed this activity to be of the ∝-type and in investigations of the effect of ions on the amylase activity, demonstrated activation by both borate and chloride ions. This activation of the algal amylase was demonstrated in a different extract by Cunningham (1961) who also showed that the amylase was activated by chloride and bromide ions and inhibited by iodide and fluoride ions.

In an attempt to confirm and extend this work, the experiments described in this chapter were carried out.

Experimental

In these experiments the Cladophora extracts used were those, the preparation of which is described in Methods and Materials, where is also described the method of amylase assay.

Measurement of the amylase activity in the fractions prepared.

The activities were measured in the following digest: 5 ml. 0.1% soluble starch, 10 ml. extract solution (100 mg./12 ml. water), 1 ml. 0.5% sodium chloride, 2 ml. acetate buffer pH 5.6 and 2 ml. water, incubated at 35°C. Samples were taken at intervals and the results shown below obtained.

	Time (hr.)	0	0.5	1.0	1.5	2.0	2.5
Fraction							
I.	Extinction (E.)	4.14	4.03	3.69	3.60	3.40	3.31
	Extinction ratio (E.r.)	1.0	0.964	0.889	0.872	0.822	0.799
II.	E.	5.05	4.79	4.67	4.65	4.53	4.39
	E.r.	1.0	0.957	0.923	0.920	0.897	0.849
III.	E.	4.35	2.32	1.08	0.54	-	-
	E.r.	1.0	0.532	0.248	0.128	-	-
IIa.	E.	3.10	2.83	2.64	2.55	-	-
	E.r.	1.0	0.858	0.802	0.772	-	-

	Time (min.)	0	5	10	15	30
IIb.	E.	4.70	4.02	3.68	3.35	2.59
	E.r.	1.0	0.854	0.783	0.712	0.550

	Time (hr.)	0	0.5	0.75	1.5
C.	E.	4.78	3.58	3.16	2.37
	E.r.	1.0	0.748	0.661	0.498

Fraction	I	II	III	IIa	IIb	C.
T0.5 hr.	6*	6*	0.53	6*	0.52	1.5
Activity	0.17	0.17	1.8	0.17	1.9	0.67 units

* value obtained by extrapolation.

Confirmation of the presence of an α-amylase using a glycogen β-limit dextrin

The following two digests were prepared and incubated at 35°C. for 48 hr.

A. 20 mg. glycogen β-limit dextrin, 50 mg. extract III, 4 ml. acetate buffer pH 5.6.

B. As A. but without extract III.

After incubation, the two digests were heated to 98°C. for five minutes to inactivate the algal extract, cooled to room temperature and 4 ml. of a commercial β-amylase preparation (Wallerstein, 1.5 mg./ml. acetate buffer pH 5.6) added to each digest.

After incubation for 24 hr. the reducing power of 1 ml. samples from both digests was determined by the Somogyi method. The results obtained are expressed as ml. 0.01 N. sodium thiosulphate/1 ml. sample.

A. 1.35 ml. (duplicate results)

B. 0 ml.

These results show a 36% apparent conversion of the β-limit dextrin to maltose after the successive attack of the algal enzyme and the β-amylase preparation. This confirms the presence of α-amylase activity in the Cladophora extracts because the β-limit dextrin has been degraded randomly by the algal extract allowing further degradation by the β-amylase to take place. Had the algal amylolytic activity been due to a β-amylase, the extract would have been unable to degrade the limit dextrin and the final reducing power measurements for digests A and B would have both been zero.

Examination of the stability of the amylase activity at 2°C.

Coupled with the basic stability experiment, the effect of added boric acid was investigated in view of previous reports that borate ions had an activating effect on Cladophora amylase (Duncan, 1956; Cunningham, 1961).

The boric acid solution used (0.2783 g./100 ml.) was such that 2 ml. in a digest of 20 ml. would give the optimum borate ion concentration of 45×10^{-4}M. determined by Duncan.

The two following solutions were prepared and kept at 2°C.

I. 300 mg. extract C., 25 ml. acetate buffer pH 5.6, 10 ml. water.
II. 300 mg. extract C., 25 ml. acetate buffer pH 5.6, 3.5 ml. boric acid solution, 6.5 ml. water.

The initial activity of the amylase and that after 22 hr. was determined by means of the following digests:-

1. 5 ml. 0.05% soluble starch, 10 ml. solution I, 5 ml. water.
2. 5 ml. 0.05% soluble starch, 10 ml. solution II, 5 ml. water.
3. 5 ml. 0.05% soluble starch, 10 ml. solution I, 1 ml. boric acid solution, 4 ml. water.

The boric acid added to digest 3. was such that the concentration of borate ions in digests 2. and 3. was the same.

Initial activity.

	Incubation time (hr.)	0	0.5	1.0	1.5
Digest					
1.	E.	4.93	3.87	3.07	2.70
	E.r.	1.0	0.784	0.622	0.548
2.	E.	4.93	3.97	3.16	2.82
	E.r.	1.0	0.804	0.640	0.562
3.	E.	4.93	4.00	3.27	2.88
	E.r.	1.0	0.810	0.661	0.584

From a plot of these results it is apparent that there is no significant difference in any of the digests. Mean T0.8 hr.: 0.5; activity 2.0 units.

After 22 hr.

	Incubation time (hr.)	0	0.5	1.0	1.5
Digest					
1.	E.	5.00	4.64	4.39	4.08
	E.r.	1.0	0.928	0.879	0.817
2.	E.	5.00	4.49	4.19	3.96
	E.r.	1.0	0.898	0.838	0.792
3.	E.	5.00	4.60	4.36	4.16
	E.r.	1.0	0.920	0.871	0.831

The results from digests 1. and 3. are not significantly different, thus demonstrating the absence of any activating effect by the borate ion. T0.8 hr. 1.78; activity 0.56 units. The result from digest 2. was T0.8, 1.33; activity, 0.75 units. Thus the amylase has been shown to lose 72% of its activity in 22 hr. at 2°C. In the presence of borate ions, the loss of activity was 62% representing a weak stabilising effect of the borate ions on the amylase activity.

Examination of the effect of borate ions on amylase stability at room temperature.

The undernoted solutions were prepared using an extract solution containing 500 mg. extract C in 50 ml. acetate buffer pH 5.6, and kept at room temperature. Samples were removed at intervals and the amylase activity measured by means of digests similar to those used in the previous experiment, half volumes being used throughout.

I. 30 ml. extract solution, 10 ml. water.

II. 15 ml. extract solution, 2 ml. boric acid solution, 3 ml. water.

In all the amylase assays carried out initially and after 6 and 25 hr. the extinction results for digests 1., 2. and 3. were equivalent and only mean extinction ratios are quoted.

Time (hr.)	0	0.25	0.5	0.75	1.0	1.3
Initial activity						
1.	4.95	4.37	3.75	3.52	-	-
2.	4.95	4.37	3.78	3.51	3.25	-
3.	4.95	4.33	3.95	3.60	-	-
E.r.	1.0	0.878	0.768	0.717	0.657	-
After 6 hr.						
1.	5.02	-	4.32	-	4.02	3.76
2.	5.02	-	4.42	-	4.01	3.73
3.	5.02	-	4.35	-	4.07	3.80
E.r.	1.0	-	0.869	-	0.800	0.750
After 25 hr.						
1.	4.85	-	4.24	-	4.04	3.75
2.	4.85	-	4.33	-	4.13	3.99
3.	4.85	-	4.33	-	4.00	3.74
E.r.	1.0	-	0.887	-	0.837	0.788

TO.8 hr. initially, 0.5, after 6 hr. 1.0 and after 25 hr. 1.4. The respective activities were 2.0, 1.0 and 0.71 units. Thus the amylase has been shown to lose 50% of its activity within 6 hr. at room temperature and 65% after 25 hr. There appears to be little difference in rates of inactivation at room temperature and at 2°C. but the stabilising effect of added borate ions at 2°C. is not evident at room temperature.

Attempted demonstration of the reported activation of amylase activity by borate ions.

Investigation of the activity of extract C in acetate and B.D.H. buffer.

Two digests were prepared containing 8 ml. 0.5% soluble starch, 10 ml. extract solution (200 mg./25 ml. water), 12 ml. water and 10 ml. buffer pH 5.6, one with acetate and the other with B.D.H. buffer. 5 ml. samples were withdrawn at intervals and the reducing power determined by the Somogyi method. The results are expressed as ml. 0.01 N sodium thiosulphate/5 ml. digest sample.

Reducing power after (hr.)	21	32	45
acetate buffer	1.45	2.13	2.55
B.D.H. buffer	1.48	1.92	2.34

The B.D.H. buffer obviously has no activating effect but there is a slight deactivating effect of doubtful significance.

Investigation of the activity of extract III in acetate and B.D.H. buffer.

Two digests were prepared containing 5 ml. 0.1% soluble starch, 10 ml. extract solution (100 mg./12 ml. water) 1 ml. 0.5% sodium chloride, 2 ml. water and 2 ml. buffer pH 5.6, one with acetate and the other with B.D.H. buffer, and incubated at 35°C. The amylase activity was determined by the iodine staining technique.

Incubation time (min.)		0	10	15	20	30	40
Acetate	E.	4.53	3.57	3.32	2.88	2.58	2.07
	E.r.	1.0	0.787	0.731	0.636	0.571	0.457
B.D.H.	E.	4.53	3.64	3.44	3.16	2.57	2.13
	E.r.	1.0	0.803	0.758	0.697	0.567	0.469

Again there is no significant difference between the results obtained for these digests. T0.5 hr. is 0.6 and the activity 1.67 units.

Examination of the effect of borate ions by iodine staining and reductiometric methods.

The two following digests were prepared and incubated at 35°C.

1. 4 ml. 0.5% soluble starch, 5 ml. extract solution (100 mg. extract C/12 ml. acetate buffer pH 5.6) 7 ml. water.
2. As above with 1 ml. water replaced by 1 ml. boric acid solution previously prepared.

After 24 hr. 2 ml. samples were removed, added to 10 ml. iodine/potassium iodide solution used in the standard amylase assay procedure, and made up to 50 ml. with water. The extinctions were measured using a yellow-green (626) and a red (608) filter with the E.E.L. colorimeter.

5 ml. samples were also taken from the enzyme digests and their reducing power determined by the Somogyi method.

Extinctions using filter		626	608
Initially		7.68	5.58
after 24 hr.	1.	4.89	2.72
	2.	4.84	2.68

Again there is no significant difference in the results obtained with different buffers. It is of interest to note that the apparent hydrolysis of the substrate, as measured by the extinction ratio, as well as the actual extinction, is dependent on the filter used. E.r. (626) is 0.634 and E.r. (608) is 0.484. The reducing power of the 5 ml. samples taken, expressed as ml. 0.01 N sodium thiosulphate were: 1. 3.30 and 2. 3.27. These results also demonstrate the absence of any effect due to added borate ions.

Examination of the effect of borate ions on the maltase activity.

As it had been shown by the experiments described above that borate ions had no effect on the amylase activity of Cladophora extracts it was thought possible that the activation effect reported by Duncan, as determined by reductiometric methods, might have been due to activation of the maltase activity also present in the algal extracts causing an increased glucose release in the digest which would upset the determination of apparent maltose resulting from α-amylolysis.

To test this hypothesis the two following digests were prepared and incubated at 35°C.

1. 5 ml. maltose solution (8 mg./ml.), 10 ml. extract solution (100 mg. extract/12 ml. acetate buffer pH 5.6), 5 ml. water.

2. As 1. with 2 ml. boric acid solution previously prepared in place of 2 ml. water.

1 ml. samples were taken after 23 hr. and their reducing power measured by the Somogyi method. The reducing power of the undernoted controls was also determined.

I. water blank.

II. 0.5 ml. extract solution (100 mg./12 ml. acetate buffer pH 5.6).

III. 1.0 ml. maltose blank (5 ml. maltose solution 8 mg./ml., 15 ml. water), 0.5 ml. extract solution (as above).

IV. 1.0 ml. maltose blank (as above).

The results expressed as ml. 0.01 N. sodium thiosulphate were:

Sample	digest 1.	digest 2.	controls I	II	III	IV
Titre	4.99) 4.90)	4.92) 4.91)	8.77	8.74	5.13	5.19

These results show that the extract solution has no significant reducing power. The difference between the mean results for digests 1. and 2. 0.03 ml. is of the order of the experimental error and demonstrates that borate ions have no effect on the algal maltase activity.

Examination of the effect of halide ions on the amylase activity.

Examination of the effect of chloride, bromide and iodide ions.

Digests containing 5 ml. 0.05% soluble starch, 10 ml. extract solution (100 mg. extract C./12 ml. water) 2 ml. acetate buffer pH 5.6 and 3 ml. 0.1 M. sodium halide or 3 ml. water, were incubated at 35°C. and amylase activity measured in the usual way.

Incubation time (hr.)		0	0.25	0.5	0.75	1.0
Digest						
Water 1.	E.	4.63	4.06	3.74	3.50	3.33
	E.r.	1.0	0.876	0.808	0.753	0.719
Bromide 1.	E.	4.63	3.90	3.53	3.31	3.02
	E.r.	1.0	0.843	0.763	0.693	0.651
Water 2.	E.	4.80	4.07	3.82	3.55	3.48
	E.r.	1.0	0.848	0.794	0.739	0.724
Bromide 2.	E.	4.80	4.02	3.72	3.40	3.18
	E.r.	1.0	0.836	0.774	0.708	0.646
Iodide	E.	4.80	4.06	3.65	3.41	3.24
	E.r.	1.0	0.844	0.760	0.710	0.675
Water 3.	E.	4.42	4.04	3.63	3.36	3.08
	E.r.	1.0	0.914	0.822	0.761	0.699
Chloride	E.	4.42	3.96	3.46	3.00	2.63
	E.r.	1.0	0.896	0.783	0.680	0.595

Mean results, T0.6 hr. were: water 1.44; bromide 1.12; iodide 1.25 and chloride 0.98.

Taking the water result as 100% activity the activation effects were: chloride 46%; bromide 28%; iodide 15%.

Examination of the effect of fluoride compared with that of chloride ions.

Digests were prepared as in the previous experiment and incubated at 35°C. Amylase activity was measured in the usual way.

Incubation time (hr.)		0	0.25	0.5	1.0	1.5
Digest						
Water	E.	4.82	4.11	3.52	2.92	2.58
	E.r.	1.0	0.852	0.732	0.607	0.536
Chloride	E.	4.82	3.93	3.28	2.64	2.26
	E.r.	1.0	0.816	0.681	0.548	0.469
Fluoride	E.	4.82	4.25	4.11	3.84	3.58
	E.r.	1.0	0.882	0.853	0.798	0.742

TO.5 hr.: water 1.84; chloride 1.25; fluoride 3.67.

Taking the water result as 100% activity then the chloride ions have caused a 47% activation and the fluoride ions 50% inhibition.

Investigation of the effect of borate ions in the presence and absence of chloride ions.

Four digests were prepared containing 5 ml. 0.1% soluble starch, 10 ml. extract solution (400 mg. extract C. in 50 ml. acetate buffer pH 5.6) and, 1. 5 ml. water; 2. 2 ml. boric acid solution previously prepared, 3 ml. water; 3. 2 ml. 0.1 M. sodium chloride, 3 ml. water; 4. 2 ml. boric acid solution, 2 ml. 0.1 M. sodium chloride, 1 ml. water; and incubated at 35°C. Amylase activity was determined in the usual way.

Incubation time (hr.)		0	0.33	0.67	1.0	1.33
Digest						
1.	E.	7.22	6.53	6.00	5.68	5.15
2.	E.	7.22	6.67	6.02	5.65	5.22
Mean E.r.		1.0	0.922	0.833	0.782	0.722
3.	E.	7.22	6.49	5.88	5.33	4.82
4.	E.	7.22	6.46	5.79	5.28	4.89
Mean E.r.		1.0	0.893	0.808	0.734	0.673

TO.8 hr. 1. and 2. 0.88; 3. and 4. 0.70.

It is obvious that the results are divided into two pairs indicative of the absence or presence of added chloride ions. The added borate ions have no effect whether chloride is present or not.

Investigation of the effect of calcium ions on the amylase activity.

The two undernoted solutions were prepared and kept at 36°C.

I. 1 g. extract C in 120 ml. acetate buffer pH 5.6, 60 ml. water.

II. 0.5 g. extract C in 60 ml. acetate buffer pH 5.6, 30 ml. calcium acetate 0.03 M.

Samples of these solutions were removed at intervals and examined for amylase activity in the usual way in the three digests indicated below.

1. 5 ml. 0.05% soluble starch, 10 ml. solution I, 5 ml. water.

2. 5 ml. 0.05% soluble starch, 10 ml. solution II, 5 ml. water.

3. 5 ml. 0.05% soluble starch, 10 ml. solution I, 3.5 ml. 0.03 M. calcium acetate 1.5 ml. water.

The quantities in digest 3. were such that it contained approximately the same amount of calcium as digest 2.

Incubation time (hr.)		0	0.25	0.50	0.75	1.00
	Digest					
Initially	1.	4.32	3.97	3.48	3.14	2.69
	2.	4.32	4.01	3.48	3.10	2.64
	3.	4.32	3.98	3.45	2.97	2.63
Mean E.r.		1.0	0.922	0.804	0.711	0.616
After 15 hr.	1.	4.10	3.93	3.89	3.87	3.73
	2.	4.10	3.92	3.80	3.77	3.68
	3.	4.10	3.84	3.79	3.78	3.68
Mean E.r.		1.0	0.949	0.934	0.928	0.901
After 19 hr.	1.	4.39	4.23	4.07	4.13	3.98
	2.	4.39	4.18	4.10	4.16	3.91
	3.	4.39	4.23	4.06	4.03	4.00
Mean E.r.		1.0	0.957	0.927	0.931	0.900

Incubation time (hr.)		0	0.5	1.0	1.5	2.0	2.5
After 39 hr.	1.	4.48	4.18	4.09	3.99	3.85	3.69
E.r.		1.0	0.933	0.913	0.891	0.859	0.823

T0.8 hr. initially 0.53; 15 hr. 2.46; 19 hr. 2.46; 39 hr. 3.1.

Activities 1.88; 0.41; 0.41; 0.32 units.

It is immediately apparent from these results that calcium has no marked effect either as an activating or as a stabilising agent, there being no significant difference in the results with or without added calcium. The already demonstrated instability of the amylase is also evident.

Investigation of the effect of ethylene diamine tetra-acetic acid (EDTA) on the algal amylase activity.

∝-Amylases from plant and animal sources are believed to be calcium containing metalloenzymes (Fischer and Stein, 1960). Treatment of such amylase solutions with a metal-complexing agent, for example EDTA, causes inhibition of the enzyme by removal of this calcium necessary to the enzymic activity.

Two digests were prepared containing 5 ml. 0.05% soluble starch, 10 ml. extract solution (100 mg. extract C./12 ml. acetate buffer pH 5.6) and 1. 5 ml. water; 2. 2 ml. 0.1 M. EDTA, 3 ml. water. These digests were incubated at 35°C. and the amylase activity assayed.

Incubation time (hr.)		0	0.5	1.0	1.5
Digest					
1.	E.	4.86	4.20	3.96	3.75
	E.r.	1.0	0.864	0.814	0.771
2.	E.	4.86	4.43	4.38	4.27
	E.r.	1.0	0.912	0.902	0.878

TO.8 hr. 1. 1.17; 2. 4.0.

These results represent an apparent deactivation of 70% by the EDTA. This suggests that the EDTA has complexed and withdrawn from the amylase a metallic ion, which is necessary for its activity. If the algal amylase is similar to those from other sources, the metallic ion would be calcium.

Investigation of glutathione and albumin as possible activating or stabilising agents.

Both glutathione and albumin were reported to have a stabilising effect on β-amylase by Walker and Whelan (1960). Solutions I-IV were prepared and kept at room temperature. The concentrations of the glutathione and albumin in solutions used were such that a final concentration of 0.5 mM. glutathione and 0.05% albumin was obtained in the 25 ml. enzyme solutions.

I. 20 ml. extract solution (800 mg. extract C./100 ml. acetate buffer pH 5.6) 5 ml. water.

II. 20 ml. extract solution, 3 ml. water, 2 ml. glutathione solution.

III. 20 ml. extract solution, 3 ml. water, 2 ml. albumin solution.

IV. 20 ml. extract solution, 1 ml. water, 2 ml. glutathione solution, 2 ml. albumin solution.

Samples were removed at zero time and after 20 hr. and amylase activity measured in digests containing 5 ml. 0.05% soluble starch, 10 ml. enzyme solution I, II, III or IV, and 5 ml. water.
E values are given in the following tables.

Initial activity

Incubation time (hr.)		0	0.33	0.67	1.0
Digest	I	4.77	4.21	3.87	3.48
	II	4.77	2.63	1.98	-
	III	4.77	4.38	3.75	3.30
	IV	4.77	4.33	3.78	3.46

The values for I, III and IV are not significantly different. The results of II are shown to be anomalous by the experiment described below.

Mean E.r. for I, III and IV	1.0	0.895	0.793	0.710

T0.8 hr. 0.65; activity 1.54 units.

After 20 hr.

Incubation time (hr.)		0	0.33	0.67	1.0
Digest	I	4.86	4.47	4.28	3.97
	II	4.86	4.48	4.27	4.01
	III	4.86	4.48	4.36	4.19
	IV	4.86	4.57	4.37	4.13

These extinction values are divided into two pairs of equivalent results.

Mean E.r. I and II	1.0	0.919	0.878	0.821
III and IV	1.0	0.929	0.898	0.854

T0.8 hr. I and II, 1.77; III and IV, 2.28.

Activity 0.56: 0.44 units.

From these results it is apparent that glutathione has no effect on the stability of the algal amylase. Albumin too has no stabilising effect; rather it exerts a deactivating effect of approximately 25%.

Investigation of the apparent glutathione activation in the initial digest II above.

Four digests were prepared containing 5 ml. soluble starch, 10 ml. extract solution (100 mg. extract C./12 ml. acetate buffer pH 5.6) and 0, 1, 2 and 3 ml. glutathione solution made up to 5 ml. with water.

Incubation time (hr.)		0	0.33	0.67
Digest	1.	4.42	3.96	3.46
	2.	4.42	3.92	3.46
	3.	4.42	3.96	3.48
	4.	4.42	3.90	3.46

It is obvious from these results that glutathione has no activation effect and that therefore the results obtained in digest II above were in error.

Discussion

∝-Amylase activity has been shown in the extracts of Cladophora rupestris examined.

Activation of the amylase by borate ions has been reported by Duncan (1956) and by Cunningham (1961) in different algal extracts but extensive attempts to demonstrate this effect in the presently available extracts have been unsuccessful.

Duncan, by reducing power measurements, showed that the amylase activity was increased to almost 300% of the initial activity by the addition of boric acid to a final concentration of 4.5×10^{-3}M. Cunningham, on the other hand was only able to demonstrate 20% activation, as determined by iodine staining methods, with a final borate ion

concentration of 7×10^{-3}M. Increasing the concentration to 21×10^{-3}M. he demonstrated a slight inhibitory effect. By reducing power measurements he demonstrated a 40% activation in 5 and 8×10^{-3}M. boric acid and 24% inhibition in 16×10^{-3}M. boric acid. The substantial differences in the activation effects demonstrated in these two extracts and the complete absence of borate ion effect in the present work suggests that this effect is dependent on the preparation of the algal extract but the manner of this dependence is not obvious.

Halide ions have a considerable effect on the activity of the algal amylase. Chloride, bromide and iodide exert an activation effect decreasing in that order. This is similar to the effect of these ions on the amylase from mammalian tissues reported by Myrback (1926) who also reported that fluoride ions had no marked effect. This contrasts with the substantial inhibitory effect of added fluoride on the algal amylase. Cunningham (1961) reported relatively weak activation of the algal amylase by chloride and bromide ions and weak inhibitory effect by iodide and fluoride ions. In his experiments the final halide ion concentration was 3×10^{-2}M. compared with a final concentration of 1.5×10^{-2}M. in the present experiments. The differing activation and inhibitory effects are probably due to this variation in concentration (cf. effect of concentration on the borate ion effect mentioned above).

α-Amylases generally are believed to be metalloenzymes containing calcium (Fischer and Stein, 1960). Although added calcium had no effect on the algal amylase the addition of EDTA caused substantial inhibition of the amylase activity, suggesting that the algal amylase falls within the general pattern, the EDTA having removed the calcium from the enzyme causing its deactivation. The inability of added calcium to affect

the algal amylase is in contrast with the stabilising effect of calcium on barley malt ∝-amylase reported by Cunningham et al (1960). One may deduce from this that if calcium is present, it is strongly bound to the enzyme molecule. In this property the algal amylase resembles microbial and mammalian amylases (Fischer and Stein, 1960).

The use of glutathione and human serum albumin was reported by Walker and Whelan (1960) as stabilisers for sweet potato β-amylase in solution. These compounds have been shown to have no stabilising effect on the algal ∝-amylase solutions. It is probable that the observed stabilising effect on β-amylase solutions is due to the presence in these stabilisers of -SH groups which are also an integral part of the β-amylase molecule, and therefore these compounds are probably specific stabilisers for β-amylase preparations (Englard et al 1951).

Summarising the results obtained, it has been shown that the present algal extract ∝-amylase is unaffected by added borate ions, in contrast with other extracts. Halide ions are activators, with the exception of fluoride ions which exert an inhibitory effect. This latter property is in contrast with other ∝-amylases. The algal amylase is apparently within the general pattern of calcium containing metalloenzymes but appears to be more closely related to microbial and mammalian amylases than to plant amylases as typified by barley malt ∝-amylase.

The ∝-amylase from Cladophora rupestris has therefore distinctive properties, but the full evaluation of these may be better achieved with more highly purified and active preparations, although this will be difficult in view of the inherent instability of the enzyme and the low activity of algal extracts.

Chapter II

STRUCTURE AND ENZYMIC DEGRADATION OF RHODYMENIA PALMATA XYLAN

Polysaccharides of the xylan group, believed to be part of the cell wall hemicellulose structure (Hirst, 1955; Aspinall, 1959) have been isolated from numerous plant sources. The common structural feature of xylans extracted from land plants is a main chain of β, 1-4 linked xylose residues, with or without side chains of other sugar units.

The xylan isolated from Esparto grass (Chanda et al 1950) and that from Tamarind seeds (Savur, 1956) are exclusively made up of xylose residues linked β, 1-4. Both these xylans are reported to have a single branch point in a molecule of about 80 units. A xylan isolated from wheat straw by Aspinall and Mahomed (1954) had a linear xylose chain of approximately 45 units with a single glucuronic acid residue linked at some point on the chain. Similar xylans, containing approximately 14% of 4-O-methyl glucuronic acid, have been isolated from cherry and apple wood, and shown to have respectively one and two branch points per molecule (Dutton and Unrau, 1962).

More complex structures have been demonstrated in which other sugars are attached to the main xylose chain. The arabinoxylan isolated from wheat flour (Perlin, 1951) was shown by Ewald and Perlin (1959), using periodate oxidation techniques, to comprise the common xylose backbone with arabinose residues as randomly placed side chains. A similar structure has been reported from a xylan extracted from rye flour (Aspinall and Ross, 1963).

Even more complex structures in which the side chains are made up of a greater variety of sugar residues have been demonstrated. Examples of this are the xylans from barley husks, which contains arabinose and glucuronic acid, and perennial rye grass, which contains arabinose, glucuronic acid and galactose (Aspinall and Ross, 1963 and Aspinall et al 1963). Further examples of the complexity of hemicellulose xylans have been reviewed by Aspinall (1959).

By comparison, little is known of the xylans present in marine algae, but the available data point to a fundamental difference from those present in land plants. Mackie and Percival (1959) isolated from the green alga, Caulerpa filiformis, a xylan with a degree of polymerisation (D.P.) of 40-45, composed entirely of β, 1-3 linked xylose units. Iriki et al (1960) isolated β, 1-3 linked xylans from the cell walls of Bryopsis maxima, Caulerpa ancepa, Halimeda cuneata and Chlorodesmis formosana with average D.P.s of 50, determined by physical and chemical methods. The xylan found in Rhodymenia palmata differs in two major respects from all other xylans. Firstly it is readily extractable by, and soluble in water, which property casts considerable doubt on its being a cell wall constituent, and secondly, the molecule contains both β, 1-3 and β, 1-4 linked xylose residues.

Following the original isolation of a xylose rich polysaccharide from Rhodymenia palmata by Barry and Dillon (1940), Chanda and Percival (1950) and Barry et al (1950) concurrently reported that the xylan contained β, 1-4 and β, 1-3 linkages in the ratio 4/1. (A subsequent determination by Barry et al (1954) gave a figure of 72% β, 1-4 linkages). By methylation studies the chain length was found to be 20-21 and the

apparent D.P., by a periodate oxidation method, 39-40. These workers therefore deduced that this xylan was similar to that from Esparto grass, having one branch point per molecule.

In the work described in the previous paragraph, it was assumed, by the failure of fractionating attempts, that the molecule was homogeneous. The existence of β, 1-3 and β, 1-4 linkages in the same molecule was confirmed by Howard (1957) who isolated and characterised xylo-oligosaccharides containing both β, 1-4 and β, 1-3 linkages, after a partial hydrolysis of Rhodymenia xylan by a mixed culture of sheep rumen bacteria. Howard was unable to isolate the trisaccharide O-β-D-xylopyranosyl-(1$\rightarrow$3)-O-β-D-xylopyranosyl-(1$\rightarrow$3)-D-xylopyranose (rhodymenatriose), from the hydrolysate and therefore concluded that there were no adjacent β, 1-3 linkages in the xylan.

The present work was undertaken in order to examine the D.P. and chain length of the molecule, and to examine the possibility of adjacent β, 1-3 linkages in the molecule, with a view to investigating the xylanase activities of Cladophora and Rhodymenia extracts.

Experimental.

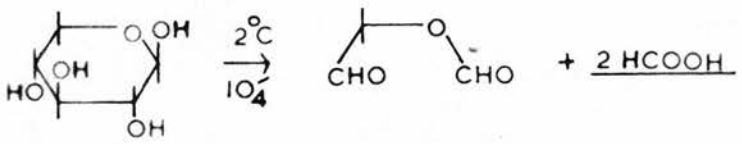
Section I

Determination of the degree of polymerisation (D.P.) and chain length by oxidation with sodium metaperiodate.

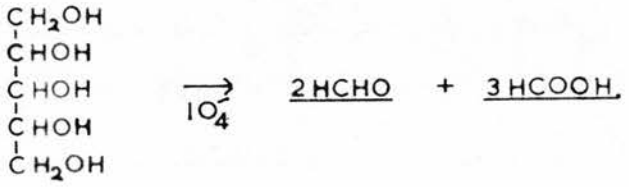
The pattern of periodate oxidation expected under the various conditions used is shown in Fig. 8. A number of experiments were carried out making use of the different results obtainable by periodate oxidation techniques.

PERIODATE OXIDATION

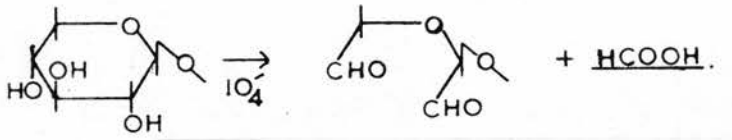
a Xylose



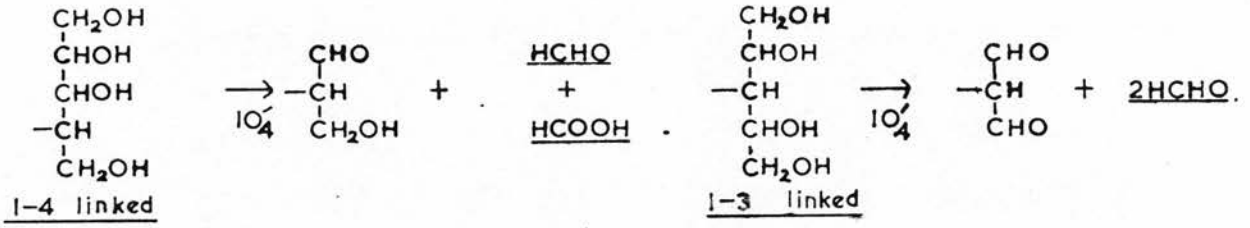
b. Xylitol



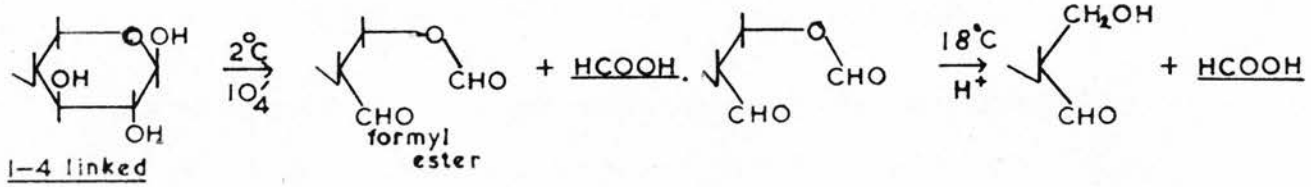
c. Non-reducing end-groups

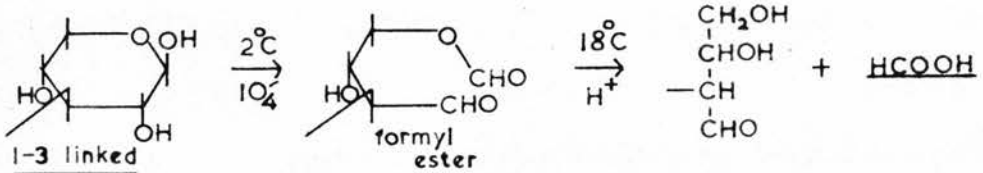


d. Xylitol end-groups



e. Xylose end-groups





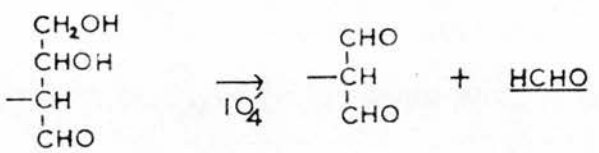


FIGURE 8

D.P. determination by estimation of the xylitol content of xylanitol D.

This method is based on the fact that xylitol should yield 2 molar proportions (mol. prop.) of formaldehyde, and xylose should yield no formaldehyde on oxidation at 2°C. (Fig. 8, a and b). Thus an estimate of the formaldehyde produced an oxidation of an acid hydrolysate of the polysaccharide alcohol will give the xylitol content, and assuming one xylitol end-group per molecule, the D.P. can be calculated.

Oxidation of xylitol at 2°C.

Xylitol solution (1 mg./ml., 1 ml.) was oxidised at 2°C. with sodium metaperiodate (3 ml., 0.3 M.) in a final volume of 15 ml. The formaldehyde released was estimated by the chromotropic acid method (Methods and Materials). Duplicate experiments were carried out.

Oxidation period (hr.)		1	2	5	24
Mol. prop. formaldehyde	I	2.0	2.0	2.0	-
	II	2.1	2.0	2.1	2.1

Oxidation of xylanitol hydrolysate and xylose control.

Xylanitol D. (250 mg.) was hydrolysed and the xylose content of the neutralised hydrolysate, determined by the Somogyi method, was 231 mg.

Oxidation solutions containing 0.3 M. periodate (3 ml.) with a final volume of 15 ml. were prepared containing xylanitol hydrolysate (23.1 mg. xylose in a 5 ml. aliquot) and xylose (25 mg.). The formaldehyde released was determined in samples taken at intervals. Extinctions (E) were measured at 570 mμ against a reagent blank.

E after (hr.) (corrected to 25 mg. xylose)	1	4
Xylose	1.30	1.44
Xylanitol hydrolysate	1.55	1.62

The mean of the difference between these results, 0.21, is equivalent to 4.7 µg. formaldehyde in a 0.5 ml. sample. In the oxidation mixtures containing 25 mg. of xylose this is equivalent to 2.35 µM. xylitol. With one xylitol per xylan molecule, the weight of xylan yielding 25 mg. xylose on hydrolysis, (132/150 x 25 mg.) is equivalent to 2.35 µM. xylan. The D.P. may therefore be calculated as 76.

In the results obtained above the yield of formaldehyde from the oxidation of xylose was much greater than expected. On the assumption that, at 2°C. xylose, like glucose, is oxidised in the pyranose form less than 0.15 mol. prop. of formaldehyde should be produced (Hough et al 1958). The following experiment shows that more than 0.3 mol. prop. was, in fact, liberated.

Oxidation of xylose and xylose/xylitol mixture at 2°C.

Xylose (10 mg.) and xylose and xylitol (10 mg. and 0.1 mg.) were oxidised in the standard 15 ml. oxidation solution at 2°C. The following results were obtained. Extinction results quoted are for 0.5 ml. samples.

Oxidation time (hr.)	0.5	1	2	4	8	24
Xylose						
E	0.67	0.82	0.89	0.92	0.97	1.03
µg. formaldehyde	15.0	18.5	20.2	20.8	21.8	23.2
mol. prop.	0.22	0.27	0.30	0.31	0.32	0.34
Xylose + xylitol						
E	0.72	-	0.96	0.97	1.00	1.07
µg. formaldehyde	16.2	-	21.6	21.8	22.4	24.0
Xylitol by subtraction						
E	0.05	-	0.07	0.05	0.03	0.04
µg. formaldehyde	1.2	-	1.4	1.0	0.6	0.8
mol. prop.	1.8	-	2.1	1.5	0.9	1.2

These results show that under the experimental conditions used, xylitol in the presence of 100 fold excess of xylose cannot be accurately estimated. Thus the D.P. obtained in the previous experiment can be taken only as an order of magnitude.

Oxidation of xylose at 18°C.

Xylose (2 mg.) was oxidised in a standard 15 ml. oxidation solution. Samples, 0.5 ml. were taken at intervals and the formaldehyde determined by the chromotropic acid method.

Time (hr.)	1	2	4	9	24	57	75	168	240
Mol. prop. formaldehyde	0.22	0.26	0.28	0.35	0.50	0.74	0.83	0.97	1.01

PERIODATE OXIDATION OF XYLOSE

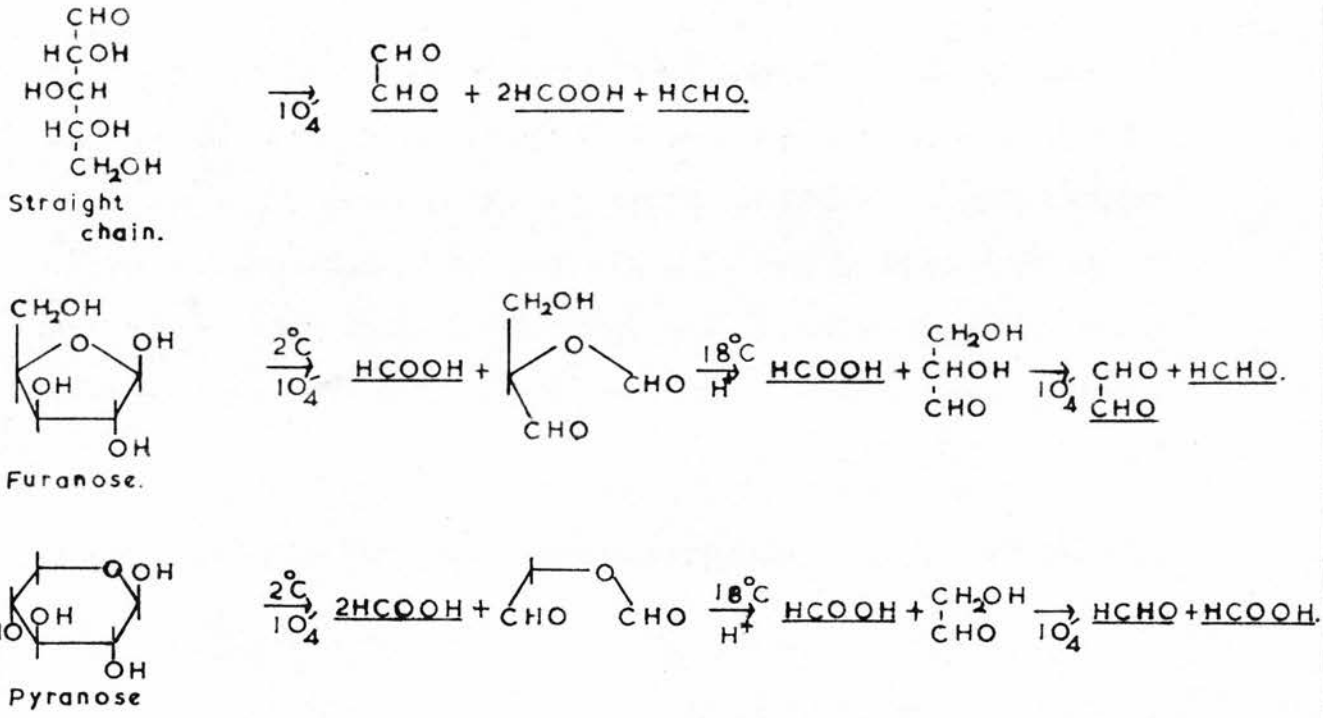


FIGURE 9

The periodate oxidation pattern for xylose in aqueous solution is not simple. Assuming the possible straight chain, furanose ring and pyranose ring structures the expected patterns are outlined in Fig. 9. The speedy release of about 0.2 mol. prop. of formaldehyde suggests a substantial contribution by the straight chain form to the equilibrium but the elucidation of the complete pattern would require a much more intensive examination than has been given here.

D.P. and chain length determination by oxidation of xylan and xylanitol samples.

Formaldehyde and formic acid will be produced only at the ends of the chains as shown in Fig. 8.

If there are A. moles of non-reducing end-groups, B. 1-4 linked reducing end-groups and C. 1-3 linked reducing end-groups then, at 18°C., oxidation of the xylanitol will yield B. + 2C. moles of formaldehyde and the xylan will yield C. moles of formaldehyde. By subtraction, B + C, the total number of reducing end-groups can be obtained and, assuming one reducing end-group per molecule, the D.P. may be calculated.

The xylanitol will yield A + B moles of formic acid at both temperatures, and the xylan A + B moles at 2°C. and A + 2B + C moles at 18°C. Again by subtraction, B + C may be obtained and the D.P. calculated. Using the figure obtained for A + B and assuming in turn that B = 0 and C = 0 the two ends of the possible range of chain lengths - xylose units per non-reducing end-group - may be calculated.

Comparison of the yield of formaldehyde from xylan and xylanitol at 18°C.

Xylan and xylanitol (25 mg. of each, both samples D.) were oxidised in the standard 15 ml. solution at 18°C. Blank solutions, to provide corrections for the charring effect produced by the chromotropic acid were prepared, in which the periodate was destroyed by adding 15 ml. M. sodium sulphite prior to the addition of 25 mg. each of xylan and xylose to separate solutions.

Extinction results

Xylose blank	0.039	0.038
Xylan blank	0.038	0.040

Period of oxidation (days)	1	2	6	7
Xylan	0.049	0.052	0.062	0.063
Xylanitol	0.113	0.111	0.112	0.113

The difference between these results, E = 0.05, is equivalent to 1.2 µg. formaldehyde in a 0.5 ml. sample. This represents 1.2 µM. of formaldehyde as the difference in formaldehyde yields from the xylan and xylanitol oxidations (25 mg. samples). As derived above there are therefore 1.2 µM. reducing end-groups, and assuming one reducing end-group per molecule the D.P. can be calculated as 157.

The number of reducing end-groups linked through C_3 is equivalent to the xylan figure less a blank for charring. This gives an extinction value of approximately 0.02 or about 40% of the total number of reducing end-groups. This figure is not significant as an absolute determination but it shows that the reducing end-groups are linked

through both C_3 and C_4 probably in the same ratio as in the rest of the molecule, that is 20-30% 1-3 linked residues (Chanda and Percival, 1950; Barry et al 1954 and determinations made in section II of this chapter).

Comparison of the yield of formic acid from xylan and xylanitol.

Oxidations of xylan and xylanitol (25 mg. each, both samples D) were carried out with 3 ml. 0.3 M. sodium metaperiodate in a total volume of 10 ml. at 2°C. and 18°C. Samples (2 ml.) were removed at intervals and the formic acid titrated with 0.00153 N barium hydroxide.

Results, titre per 2 ml. sample

Oxidation period (days)		1	2.3	4	7
Xylanitol	18°C.	0.50 ml.	0.68 ml.	0.74 ml.	0.93 ml.
	2°C.	0.30 ml.	0.73 ml.	0.70 ml.	0.88 ml.
Xylan	18°C.	0.73 ml.	0.95 ml.	0.94 ml.	1.12 ml.
	2°C.	0.66 ml.	0.73 ml.	0.74 ml.	0.91 ml.

The fact that the final figures for the xylanitol and the xylan at 2°C. are the same indicates the absence of formyl ester hydrolysis in the latter case. As derived above the difference between these results and that from the xylan at 18°C. is equivalent to the total number of reducing end-groups, B + C.

A plot of the titration figures for the oxidation of the xylanitol and the xylan 2°C. and the xylan 18°C. showed that the formic acid yield was increasing. However, the difference required remained constant after 2.3 days and this figure was taken to represent B + C.

This difference, 0.21 ml. 0.00153 N barium hydroxide for the 2 ml. samples used is equivalent to 1.05 ml. or 1.64 μM. hydroxide for a 25 mg. xylan sample. Thus 25 mg. xylan contains 1.64 μM. B + C, that is 25 mg. is equivalent to 1.64 μM. xylan and the D.P. may be calculated as 115.

A value for A + B from the xylanitol and xylan 2°C. results cannot be obtained as the formic acid yield was not constant.

Comparison of the formic acid yields from xylans M and W at 2°C. and 18°C.

Two samples of xylan (50 mg. each of samples M. and W.) were oxidised at 18°C. and 2°C. with 6 ml. 0.3 M. periodate in a final volume of 20 ml. The formic acid released was determined, on 2 ml. samples, by titration with 0.00073 N barium hydroxide.

Results: ml. 0.00073 N barium hydroxide

2°C.

Oxidation period (days)	7	10	13
Xylan M	1.70	1.68	1.70
W	0.80	0.80	0.80

18°C.

Oxidation period	1	3	5	7	9
Xylan M	1.48	1.75	2.24	2.80	3.15
W	0.75	1.05	1.50	1.95	2.30

The formic acid release from both xylans, oxidised at 18°C., increased linearly with time. This was probably due to over-oxidation, and thus invalidates any determination of the total formic acid released from the ends of the chain, A + 2B + C.

In this experiment, by using longer oxidation periods, constant results were obtained for the release of formic acid at 2°C. equivalent to A + B.

Considering this value A + B, if B = 0 then an apparent chain length, the number of xylose residues per non-reducing end-group may be calculated. If B ≠ 0 then A is less than A + B and the chain length is greater than the value calculated assuming B = 0, which is therefore to be regarded as a minimum chain length.

A + B for xylans M and W, related to 50 mg. xylan are 17.00 ml. 0.00073 N barium hydroxide, 12.4 µM., and 8.00 ml. hydroxide, 5.84 µM., respectively. These figures are equivalent to minimum chain lengths of 31 and 64 respectively.

Assuming a linear structure and the ratio B/C to be 75/25% (Chanda and Percival, 1950; Barry et al 1954; this chapter, section II) the minimum D.P. may be calculated as shown below.

$$A = B + C$$

$$\therefore A + B = 2B + C$$

$$\text{Since } C = B/3$$

$$A + B = 7B/3$$

$$\therefore B = 3(A + B)/7$$

$$\text{and } B + C = 4(A + B)/7$$

This is a minimum D.P. value since any branching would make $A > B + C$ and hence $B + C < 4(A + B)/7$, thus increasing the D.P.

Substituting the values for A + B obtained from the 2°C. oxidation experiments in the equation derived above, B + C (M) is equivalent to 9.7 ml. hydroxide, 7.1 µM., and B + C (W) to 4.6 ml. hydroxide, 3.3 µM., each for 50 mg. xylan.

Thus 50 mg. xylans M and W represent 7.1 and 3.3 μM. xylan and the apparent D.P.s may be calculated as 53 and 115 respectively.

Conclusions

From the experiments described above, approximate values have been obtained for the D.P. of xylan D of 115-157. Minimum values for the D.P. of xylans M and W have been calculated, assuming a linear structure, as 53 and 115 respectively.

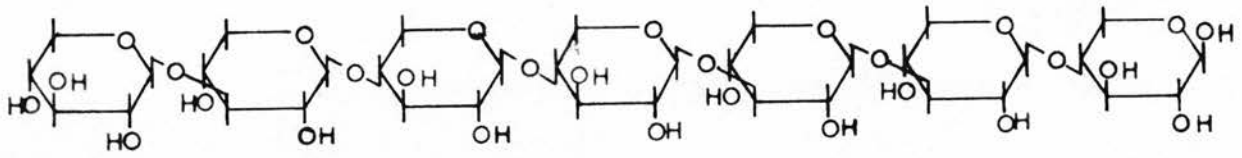
Minimum chain lengths have been calculated for xylans M and W as 31 and 64 respectively. These figures compare with 15-18 quoted by Barry et al (1954) for an average chain length obtained from formic acid release during periodate oxidation of their xylan sample and 20-21 quoted by Chanda and Percival (1950) for the chain length of a different xylan sample determined by methylation studies.

The variation in apparent molecular size in the different samples is probably due to degradation during extraction since xylan W, extracted in butanol-water mixtures, is markedly larger than the other, acid-extracted samples.

The substantial difference between the minimum chain lengths calculated and the apparent D.P. determined for xylan D. suggests a small degree of branching in the xylan molecule, but confirmation of this must await the application of more accurate methods of determining the D.P.

SMITH DEGRADATION

Rhodymenia xylan (idealised representation)



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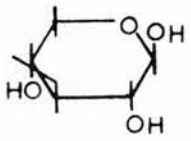
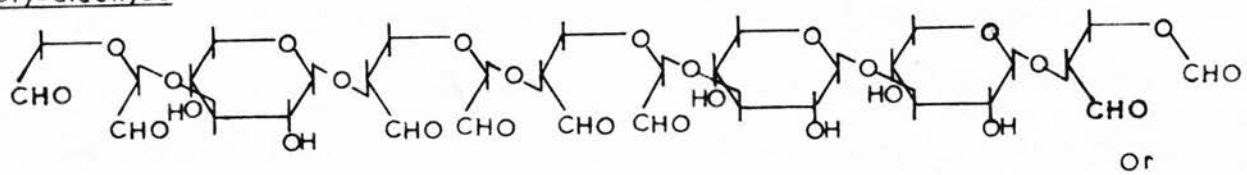


periodate oxidation

Poly-aldehyde



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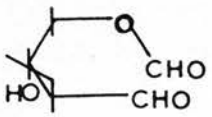
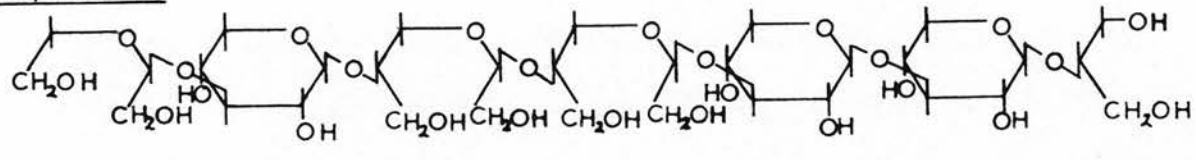


borohydride reduction

Poly-alcohol



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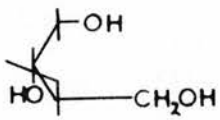
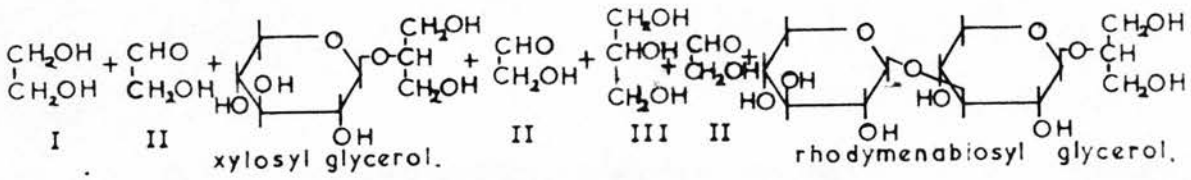
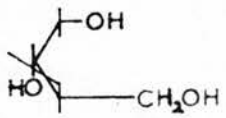


mild acid hydrolysis



I II xylosyl glycerol. II III II rhodymenabiosyl glycerol.

Or



threitol

I glycol
II glycollic aldehyde
III glycerol

FIGURE 10

Section II

'Smith degradation' of Rhodymenia palmata xylan.

This method, in which the polysaccharide molecule is subjected to periodate oxidation followed by reduction of the oxidised product, the poly-aldehyde, with potassium borohydride and mild acid hydrolysis of the reduced product, the poly-alcohol, was first reported by Smith and his collaborators (Goldstein et al 1959) who applied it to oat glucan, a polysaccharide containing both β, 1-4 and 1-3 linked glucose residues. Other examples of the application of this method are to be found in the investigation of the structure of cherry and apple wood xylans by Dutton and Unrau (1962) and in the examination of the distribution of the arabinose side chains in rye flour and barley husk xylans by Aspinall and Ross (1963). The advantage of the method is that the mild acid hydrolysis should selectively cleave the acetal linkages, leaving the glycosidic linkages intact. An example of the expected degradation pattern, that for Rhodymenia palmata xylan, is outlined in Fig. 10. Isolation and identification of the glycosides in the hydrolysate, containing one, two or more sugar units will give direct evidence of the relative positions of periodate resistant units in the original polysaccharide.

As can be seen from Fig. 10, the presence of adjacent 1-3 linkages in Rhodymenia xylan would be indicated by the isolation of rhodymenabiosyl-glycerol after a 'Smith degradation'. The isolation of 2-O-β-D xylosyl-glycerol (xylosyl-glycerol) as the sole xylose containing fragment would indicate that the β, 1-3 linkages were singly placed throughout the molecule.

The experiments reported below were undertaken in an attempt to resolve the problem of the positioning of the 1-3 linkages in the xylan molecule.

Experimental

Prior to a large scale preparation of the xylan poly-alcohol a number of exploratory experiments were carried out.

Xylan D (1 g.) was oxidised with a two-fold excess (3.3 g.) of sodium metaperiodate in a final volume of 175 ml. at room temperature for three weeks. Inorganic material and fragments of polysaccharide were removed by dialysis against numerous changes of distilled water until negative tests were obtained for periodate. The dialysed solution was freeze dried to yield 600 mg. white powder.

The poly-aldehyde (600 mg.) was treated with potassium borohydride, (200 mg.) in a total volume of 50 ml. for 24 hr. at room temperature. Further borohydride (100 mg.) was added and after 24 hr. the solution was dialysed against running water and freeze dried to give 400 mg. of poly-alcohol.

Qualitative experiments showed that even in 0.5 N sulphuric acid at 2°C. for 24 hr. free xylose was present in the hydrolysate as identified by paper chromatography.

Charcoal/Celite fractionation of a hydrolysate of the poly-alcohol, (100 mg. in 3 ml. 0.5 N sulphuric acid at 2°C. for 5 days) showed the presence of a non-reducing component with R_x 1.0 in 6/4/3 and 10/4/3. In view of the reported similarity in chromatographic mobilities of xylose and xylosyl-glycerol (Aspinall and Ross, 1963) this component was tentatively identified as xylosyl glycerol and a large scale preparation of xylan poly-alcohol started.

Xylan W (8 g.) was treated according to the procedure set out above yielding 3.2 g. of poly-alcohol which was hydrolysed for 3 hr. in 2 N. sulphuric acid at room temperature (Aspinall and Ross, 1963).

Paper chromatographic examination of the hydrolysate showed the presence of free xylose. After charcoal/celite fractionation, xylobiose was identified in the fractions by paper chromatography. This indicated that the periodate oxidation of the xylan was incomplete, thus invalidating any results obtained from this experiment, which was therefore abandoned.

In order to determine the period of oxidation required to effect complete breakdown of all the ∝-glycol groups susceptible to periodate, a small scale oxidation was carried out and the reduction of periodate followed to a constant value.

Xylan W (55 mg.) was oxidised at 2°C. by 3 ml. 0.3 M. sodium metaperiodate in a total volume of 15 ml. Samples (1 ml.) were removed at intervals, diluted to one litre and the extinction of this solution measured at 222.5 mμ. (see Methods and Materials). A periodate calibration graph was prepared in which 100% periodate gave an extinction of 0.601 and 0% periodate 0.086.

Results

Time (days)	1	2	6	8	10	14	18	21
Extinction	0.482	0.468	0.446	0.433	0.423	0.415	0.412	0.413
% periodate reduced	23	26	30	33	35	36	37	37

Assuming 100% 1-4 linkages in the xylan and the resultant reduction of one mole of periodate for each xylose residue one can calculate the weight of periodate required to effect complete oxidation to be 89.3 mg. As 3 ml. 0.3 M. periodate contains 192.6 mg., 89.3 mg. represents a 46.4% reduction of the available periodate. The experimental result of 36.5%, equivalent to 79% of the theoretical shows that only 79% of the

xylose residues are susceptible to periodate oxidation, that is, only 79% of the xylose residues are linked through the 4 position. This compares with figures of 80% and 72% found respectively by Chanda and Percival (1950) and Barry et al (1954) for different xylan samples.

The above results show that an oxidation period of not less than two weeks is required when approximately two-fold excess of periodate is used at 2°C.

Preparation of the xylan poly-alcohol

Xylan W (20 g.) was oxidised with a two-fold excess (65 g.) of sodium metaperiodate in a final volume of five litres at 2°C. for 20 days. After this period the reduction of periodate was determined and the following extinctions obtained: 100% periodate 0.613, oxidation experiment 0.424, 0% periodate 0.086. The experimental result is equivalent to a 36% reduction of the available periodate, or 72% of the theoretical figure calculated on the basis of a 100% 1-4 linked xylan. This result is in agreement with the results quoted above.

The reaction was stopped by precipitating the remaining periodate and iodate, formed in the oxidation, with saturated barium hydroxide solution until the solution gave a positive test for barium. The precipitate was removed by centrifugation and the solution passed through a column (35 x 6 cm.) of I.R. 120 (H) containing 500 g. of resin. The column was washed with one litre of water and allowed to drain. As the eluate from this column was weakly acid, pH 2.7, it was neutralised by passing it through a column (40 x 4 cm.) of I.R. 45 (OH) containing 200 g. of resin. The column was washed with one litre of water and allowed to drain. The neutral eluate was treated with a large excess (10 g.) of potassium borohydride.

After four days, residual borohydride was destroyed and potassium ions removed by passing the solution through the regenerated I.R. 120 column. The eluate from this column, about seven litres was concentrated and boric acid removed by repeated evaporation with methyl alcohol until negative tests were obtained for borate. All concentration and evaporation was done with a bath temperature of about 40°C. The remaining syrup, dried over phosphorus pentoxide, in vacuo, weighed 4.4 g.

Paper chromatographic examination in 6/4/3 using both silver nitrate and aniline phthalate sprays showed the syrup to contain xylose and xylitol as well as polymeric material. The presence of these monosaccharides indicates that the poly-aldehyde and poly-alcohol molecules have been partially degraded during their preparation, probably by the I.R. 120 resin.

In order to obtain the poly-alcohol free from mono- and oligo-saccharides the syrup was repeatedly fractionated on a 1000 ml. column (80 x 4 cm.) of Sephadex G-25. Fractions (10 ml.) were collected and examined by paper chromatography. No complete separation of polymeric from mono- and oligo-meric material was obtained, but by arbitary division according to the paper chromatographic information and re-chromatography on the Sephadex column, 1.5 g. poly-alcohol free from small oligosaccharides and 2.7 g. low molecular weight material free from polymeric material were obtained.

Fractionation of the low molecular weight fraction, LMW.

Paper chromatography in 10/3/3 showed this fraction to contain compounds with R_x values of 0.57, 0.8, 1.0 and 1.4. These can be

compared with established standard values, xylobiose 0.4, rhodymenabiose and xylitol 0.8, erythritol 1.35 and glycerol 2.2. All four components give positive reactions to both silver nitrate and aniline phthalate sprays which property serves to distinguish the fastest moving spot from erythritol which does not give a positive reaction with aniline phthalate. These four components will be referred to as LMW 1, 2, 3 and 4.

This mixture was fractionated on a charcoal pad (30 g.) carried on filter paper in a 9 cm. Buchner funnel. Fractions (500 ml.) were collected and examined as described below.

Fraction	Eluant	Chromatographic and probable identity of the components	
1-3	water	LMW 2 and 3	xylose and xylitol
4-6		LMW 2, 3 and 4	rhodymenabiose, xylosyl xylitol, xylosyl glycerol and LMW 4.
7	5% ethanol	LMW 2, 3 and 4	rhodymenabiose and traces of xylosyl glycerol, xylosyl xylitol and LMW 4.
8-11		LMW 2, 3 and 4	rhodymenabiose, xylosyl xylitol, and LMW 4.
12-16	15% ethanol	LMW 1 and 2	a 'trisaccharide' (LMW 1) and LMW 2 unidentified

Fractions 1-3 were shown to contain xylose and xylitol by paper chromatography in 6/4/3.

Fraction 7 was concentrated to yield 80 mg. of syrup which was fractionated by thick paper chromatography (in 10/3/3 3 MM paper). The band corresponding to LMW 2 was eluted and re-chromatographed on thick paper to remove traces of LMW 3. Finally it was chromatographed on a small charcoal column (4 g.) to remove contaminating, non-reducing material from the thick paper. A final yield of about 8 mg. was obtained.

Complete acid hydrolysis and paper chromatographic examination of the neutralised hydrolysate showed only xylose, using both silver nitrate and aniline phthalate sprays. The D.P. determined by the phenol/sulphuric acid method (Methods and Materials) was 2. The extinction results, measuring the apparent reducing sugar content before and after reduction of the oligosaccharide with potassium borohydride, were: before reduction 0.725, 0.730; after reduction 0.364, 0.363, all extinctions being read against reagent blanks.

Chromatography in 10/3/3 gave a single reducing component with R_x 0.76 which agrees with 0.77 for authentic rhodymenabiose from the partial acid hydrolysate of Caulerpa filiformis xylan (Methods and Materials). Thus, rhodymenabiose was identified as the major component of fraction 7.

Fractions 4-6 and 8-11 were combined and chromatographed on a charcoal/Celite column (30 g. of each). The column was washed with about two litres of water to remove traces of xylose and xylitol and was eluted with 5% ethanol. Sixty 25 ml. fractions were collected and examined with the phenol/sulphuric acid reagent. The first and last ten fractions contained negligible amounts of material. No complete

separation was demonstrated by the colorimetric examination of the remaining fractions but paper chromatographic examination (in 10/3/3) showed that fractions 11-32 contained LMW 3 and 4 with only traces of LMW 2, and fractions 33-50 contained LMW 2, identified as rhodymenabiose in fraction 7 above, and small amounts of LMW 3. The combined series of fractions 11-32 and 33-50 are referred to below as fractions 1132 and 3350 respectively.

Fraction 1132 was concentrated and chromatographed in 10/3/3 on 3 MM paper. LMW 4 was rejected as an artefact of the preparative procedure, no corresponding component being demonstrable in the poly-alcohol hydrolysates examined in subsequent experiments described below. LMW 3 was eluted and chromatographed in mek/water giving a spot with R_x 0.76 (Dutton and Unrau, 1962, quote 0.76 for xylosyl-glycerol in this solvent) and a trace of xylose. This trace of xylose was removed by thick paper chromatography in mek/water. Hydrolysis of the eluted and concentrated LMW 3 gave xylitol, xylose and a trace of glycerol (in 6/4/3). Electrophoresis of LMW 3 gave one major spot M_x 0.17 and two other spots M_x 0.63 and 0.88. The major component was separated electrophoretically (250v. 3 MM paper, 22 x 7.5 cm. 2 hr.). The slow moving band was eluted, the eluate treated with I.R. 120 (H) to remove sodium ions and repeatedly evaporated with methanol to remove boric acid. Paper chromatography in 6/4/3 showed a neutralised hydrolysate of this component to contain xylose and xylitol. Thus a xylosyl-xylitol was identified as a major component of LMW 3. The presence of glycerol in the hydrolysate prior to electrophoretic fractionation and the presence of trace components shown by electrophoresis suggests the possible presence

of xylosyl-glycerol but this could not be demonstrated.

Fraction 3350 was freed from the small amount of LMW 3 by thick paper chromatography (in 10/3/3 on 3 MM.). Elution, concentration and purification by adsorption on to charcoal with subsequent elution with 5% ethanol failed to remove sufficient of the coloured impurities to permit characterisation by specific rotation. The yield was about 10 mg. Chromatographically (in 10/3/3) and electrophoretically this component of LMW 2 was indistinguishable from authentic rhodymenabiose, from the Caulerpa xylan hydrolysate, R_x 0.77 (0.77), M_x 0.6 (0.6).

Final characterisation was effected by methylation according to the procedure described in Methods and Materials. A neutralised hydrolysate of the methylated product chromatographed in 200/17/1 gave two components with R_f 0.41 and 0.77. These agree with figures of 0.42 for authentic 2:4 di-O-methyl xylose obtained by methylating part of the Caulerpa xylan hydrolysate and 0.78 for authentic 2:3:4 tri-O- methyl xylose from the same source. The ratio of the two components was determined by the method of Wilson (1959) outlined in Methods and Materials. A further sample of the neutralised hydrolysate was chromatographed in 200/17/1 and after development with aniline phthalate the spots were cut from the paper, the colour eluted and the extinctions of the solutions measured at 360 mμ. The extinction readings were: paper blank, 0.27; 2:4 di-O-methyl xylose 0.38; 2:3:4 tri-O-methyl xylose, 0.43. The ratio 2:4/2:3:4 is 0.69 which agrees with a figure of 0.74 for the ratio of 2:3/2:3:4 obtained below for a hydrolysate of methylated authentic xylobiose. The variation of these ratios from unity is probably due to the differing chromogenic properties of the di- and tri-O-methyl xyloses with respect to aniline phthalate.

This methylation experiment confirms the identification of rhodymenabiose as a major component of LMW 2.

Fractions 12-16, the 15% ethanol eluate, containing LMW 1 and the remainder of LMW 2, were concentrated and separated on thick paper (in 10/3/3 on 3 MM.).

The LMW 2 fraction, on chromatography in mek/water and 6/4/3 and on electrophoresis showed a complex mixture of substances. Xylose, xylitol and glycerol were identified on chromatography (in 6/4/3) of an acid hydrolysate. This fraction was not examined further.

LMW 1 was eluted and the apparent D.P. determined by the phenol/sulphuric acid method. Extinctions, measured at 480 mμ. were: before reduction 0.639, 0.643, 0.646; after reduction 0.421, 0.420, 0.426. These extinctions were read against a reagent blank. These results give an apparent D.P. of 2.9. This compound is henceforth referred to as the 'trisaccharide'. The 'trisaccharide' had R_x values of 0.9, 0.56 and 0.23 in 6/4/3, 10/3/3 and mek/water respectively. Chromatographed in 9/1/1 the 'trisaccharide' was virtually immobile but a trace of a component with R_x 1.2 was evident. This trace was removed by thick paper chromatography in 9/1/1. Electrophoretically the 'trisaccharide' split into three components with M_x of 0.46, 0.69 and 0.82. Complete acid hydrolysis gave xylose and a compound with R_x 2.0 in mek/water hereafter referred to as compound Z. The R_x 2.0 found for compound Z compared with 1.3 for erythritol, 1.2 for threitol and 2.5 for glycerol in the same solvent system. A trace of xylitol was also found in the hydrolysate. Partial acid hydrolysis (10 and 30 min. 0.1 N sulphuric acid 98°C.) gave the same results as the total acid hydrolysis

except that in the 10 minute hydrolysis there was still unhydrolysed 'trisaccharide' in the mixture.

Part of this 'trisaccharide' was methylated according to the procedure outlined in Methods and Materials. Paper chromatography of a neutralised hydrolysate of the methylated product in 200/17/1 showed it to contain two major components with the R_f values of 2:3:4 tri-O-methyl xylose, 0.78 (0.78 for an authentic sample) and 2:4 di-O-methyl xylose, 0.42 (0.42). Authentic 2:3 di-O-methyl xylose was obtained by methylation of xylobiose and had R_x 0.51 in this solvent system. The ratio of these two components was determined (Wilson, 1959), the extinctions, measured at 360 mμ. being: paper blank 0.338; 'trisaccharide' 2:4 di-O-methyl xylose, 0.600, 0.579, 2:3:4 tri-O-methyl xylose 0.629, 0.640; xylobiose 2:3 di-O-methyl xylose 0.828, 2:3:4 tri-O-methyl xylose 1.00. The experimental ratio di/tri-O-methyl xylose for xylobiose was 0.74, for the 'trisaccharide' 0.85. Assuming that the low xylobiose figure is due to the different chromogenic properties of the di- and tri-O-methyl xyloses towards aniline phthalate, this figure may be taken as unity. Appropriate scaling up of the 'trisaccharide' figure gives a ratio of 1.2/1. This figure is significantly removed from that of 2/1 expected from the D.P. determination and only serves to confirm the mixed nature of the 'trisaccharide' LMW 1. The only deduction which can be made from the results is that the 'trisaccharide' mixture contains rhodymenabiose residues, at least some of which are blocked at the reducing end by the non-reducing compound Z, thus causing the high D.P. result.

The low molecular weight fraction has therefore been shown to contain a mixed fraction LMW 1 containing rhodymenabiose residues, a

Poly alcohol small scale hydrolysate: Separation.

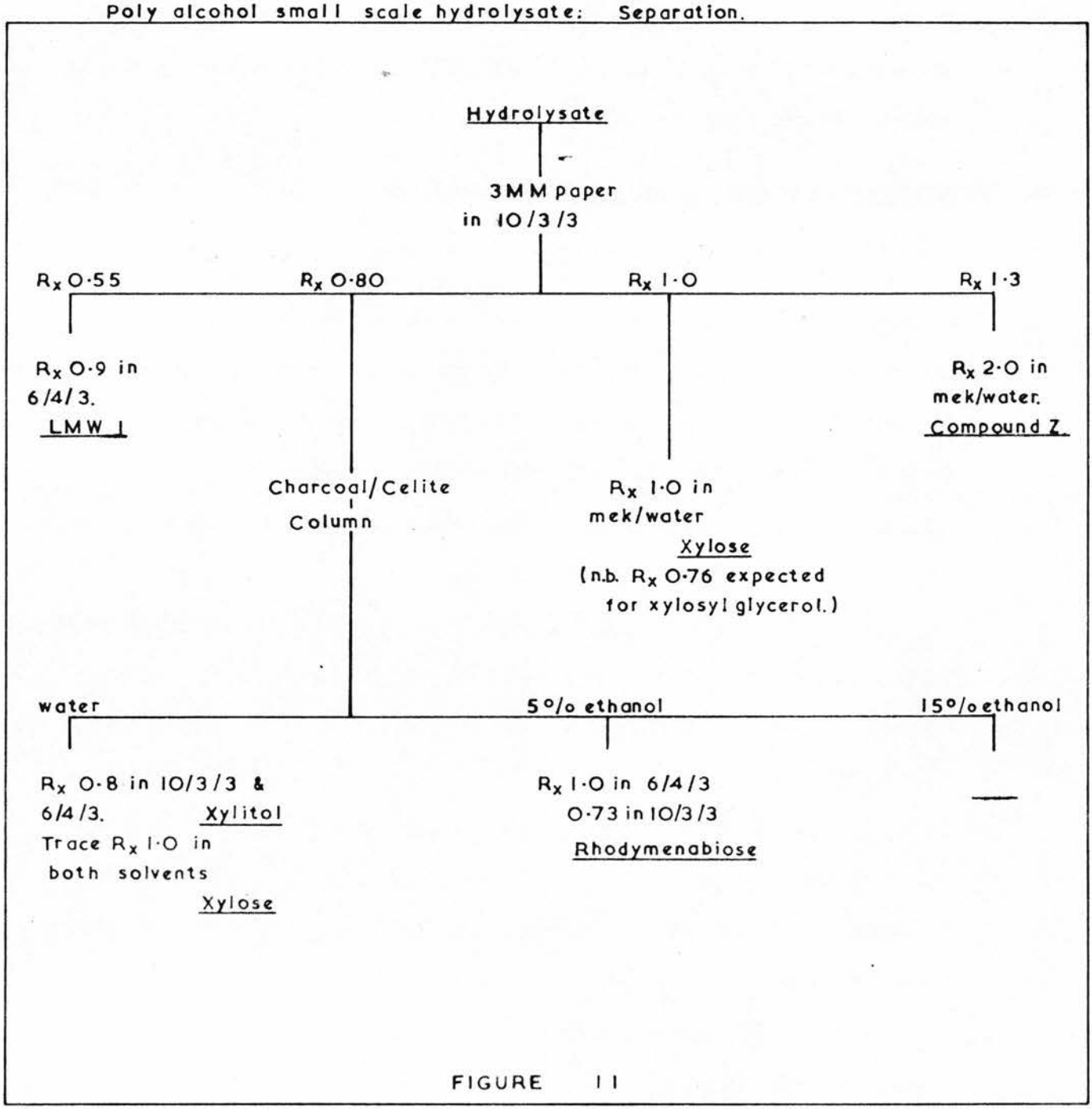


FIGURE 11

fraction LMW 2 containing xylitol, rhodymenabiose and an unidentified mixed component, a fraction LMW 3 containing xylose, xylosyl-xylitol and possibly a trace of xylosyl-glycerol and a fraction LMW 4 which was rejected as an artefact.

Examination of the poly-alcohol fraction

A preliminary hydrolysis of the poly-alcohol (60 mg.) in N sulphuric acid at room temperature was followed by paper chromatography of neutralised samples, withdrawn at intervals. The first sample, taken after thirty minutes showed the presence of free xylose. Further samples withdrawn up to three hours contained increasing amounts of free xylose and also traces of oligosaccharide material which was masked by streaking of unhydrolysed poly-alcohol down the paper.

A further sample of poly-alcohol (200 mg.) was hydrolysed at room temperature in N sulphuric acid (10 ml.) for eighteen hours in an effort to achieve complete breakdown of the acetal linkages in the molecule. A chromatogram of the hydrolysate showed heavy streaking from the starting line to beyond the position of a xylose standard. This was due in part to unhydrolysed poly-alcohol and in part to glycollic aldehyde, a product of the hydrolysis. The concentrated hydrolysate was chromatographed on a 100 ml. column (32 x 2 cm.) of Sephadex G-25. Fractions (2 ml.) were collected and examined chromatographically. Those fractions containing only polymeric material were rejected and the others combined, concentrated and separated by thick paper chromatography in 10/3/3 on 3 MM. paper. Four distinct bands were obtained. A summary of the separation and identification of these bands is given in Fig. 11.

Xylose, xylitol, rhodymenabiose, 'trisaccharide' LM 1 and compound 2 were identified chromatographically.

Large scale hydrolysis of the poly-alcohol

The hydrolysis conditions used were those under which xylosyl-glycerol is reported to be stable (Aspinall and Ross, 1963). The poly-alcohol (900 mg.) was hydrolysed (N sulphuric acid, 40 ml. 3 hr. room temperature) and the concentrated, neutralised hydrolysate chromatographed on a 600 ml. column (120 x 2.5 cm.) of Sephadex G-25. Fractions (10 ml.) were collected and examined chromatographically (in 10/3/3). Those fractions containing only polymeric material were combined, evaporated to dryness and weighed (240 mg.). This material was hydrolysed (N sulphuric acid, 12 ml., 3 hr. room temperature) and treated as above yielding 140 mg. of polymeric material. This was hydrolysed (N sulphuric acid, 16 ml., 3 hr. room temperature), neutralised and added to the combined oligosaccharide fractions from the two previous hydrolyses. This mixture was chromatographed on an Ultrasorb charcoal/Celite column (40 g. of each). The fractions listed below were collected and examined.

Fraction	Eluant	Volume (ml.)	Product(s)
1		500	Glycerol, glycollic aldehyde
2	water	500	
3		100	Xylose and xylitol
4		500	
5	2% ethanol	1500	
6		2000	
7	5% ethanol	2000	Xylosyl-glycerol (2 mg.)
8	8% ethanol		Rhodymenabiose and compounds
9	10% ethanol	5000	W and Y which are mixtures and
10	12% ethanol		have not been identified.
11	20% ethanol		'Trisaccharide' LAW 1 (5 mg.)
12	30% ethanol	3000	and an oligosaccharide fraction
			not separated.

Fraction 1 on concentration and chromatography (in 10/3/3) was shown to contain glycerol and glycollic aldehyde, the latter causing characteristic streaking of the chromatogram. Fractions 2-5 chromatographed in 6/4/3 contained only xylose and xylitol. Fraction 6 contained no significant amount of material.

Fractions 7-10 were collected initially in 25 ml. fractions and examined with the phenol/sulphuric acid reagent in an attempt to follow the elution pattern but in practice the amount of material eluted was so small that no significant guide was obtained. The fractions were recombined in the manner indicated.

Fraction 7 on concentration and chromatography (in 10/3/3) gave a single spot with R_x 1.0. Purification on thick paper (in 10/3/3) to remove Celite washed off the column yielded about 2 mg. of sugar. Chromatography in mek/water gave a single spot R_x 0.73 (Dutton and Unrau, 1962 quote 0.76 for xylosyl-glycerol in this solvent system). Electrophoresis gave a single spot M_x 0.15. Hydrolysis and chromatography of the hydrolysate in 6/4/3, 10/3/3 and mek/water gave in each case two spots with the same mobilities as xylose and glycerol. Fraction 7 was thus shown to contain a xylosyl-glycerol, probably the 2-O-β-D xylosyl-glycerol expected as a product of this Smith degradation (see Fig. 10).

Fractions 8-10 combined were separated on thick paper (in 10/3/3) into three bands which were examined separately.

The first, slowest moving band was a reducing sugar, giving a strong pink spot with aniline phthalate (cf. xylo-oligosaccharides) with R_x 0.77 (0.77) in 10/3/3. Complete acid hydrolysis gave substantially only xylose with slight traces of xylitol. The D.P. determined by the phenol/sulphuric acid method was 2.2. The extinction results read at 480 mμ against reagent blanks were: before reduction 0.654, 0.661; after reduction 0.357, 0.364. The ratio after/before is 0.55 which is equivalent to an apparent D.P. of 2.2. This experiment was carried out on samples from a solution of the whole fraction in 50 ml. Thus by using a xylose calibration graph with the phenol/sulphuric acid reagent the concentration of the solution before reduction was approximately 40 μg/ml. That is, the whole fraction contained approximately 2 mg. The remainder of the fraction after the D.P. determination was methylated and the

methylated product treated with 3% methanolic HCl at 98°C. for 18 hr. The products were found, by gas chromatography, (Aspinall, 1963) to be the methyl xylosides of 2:4 di- and 2:3:4 tri-O-methyl xylose, confirming the nature of this fraction which has thus been characterised as rhodymenabiose. Grateful acknowledgement is due to Dr. G.O. Aspinall for the gas chromatographic analysis.

The second band from the separation of fractions 8-10, designated compound W had R_x 0.9 in 10/3/3 and 0.83 in mek/water. Electrophoresis separated this compound into three spots with M_x 0.43, 0.63 and 0.84. Hydrolysis of compound W gave xylose and compound Z previously found in a hydrolysate of LMW 1. Compound W has not been examined further.

The third band from the separation of fractions 8-10, designated compound Y, gave a single spot R_x 1.0 in 10/3/3 and two merging spots R_x 0.75 and 1.0 in mek/water. Electrophoresis gave one major spot M_x 0.6 with traces of faster moving material. Chromatography of a hydrolysate of compound Y in 10/3/3 and mek/water showed only xylose, but electrophoresis showed two spots with M_x 0.55 and 1.0. Compound Y has not been further examined.

Fractions 11 and 12 combined were separated on thick paper (in 10/3/3), a series of indistinctly separated bands being obtained. Only one band was eluted and re-chromatographed twice (in 10/3/3) to remove traces of overlapping bands. From the second chromatogram the front and rear halves were separately eluted, concentrated and shown to have exactly the same electrophoretic pattern:- three spots with M_x 0.5, 0.67 and 0.82 (cf. LMW 1 above). The re-combined fractions (5 mg.)

and a hydrolysate were chromatographically (in 10/3/3, 6/4/3 and mek/water) indistinguishable from the 'trisaccharide' LMW 1 run on the same chromatograms.

The remaining bands, chromatographically slower than the band eluted above from the separation of fractions 11 and 12 were eluted as the oligosaccharide fraction (40 mg.). Chromatography of a hydrolysate showed the major component to be xylose with traces of other unidentified compounds.

Half of this oligosaccharide fraction was subjected to a small scale 'Smith degradation' in an effort to detect periodate-resistant xylose residues. As is shown in Fig. 10 xylose units substituted in position 3 are resistant to periodate oxidation and therefore the isolation of xylose from a periodate oxidation of this oligosaccharide fraction will indicate the presence of rhodymenabiose fragments within the structure of the mixture.

Oxidation was effected with excess sodium metaperiodate (3 ml. 0.3 M in a final volume of 15 ml. at 2°C.) and was complete after five days, the reaction being followed by the reduction of periodate. Extinction values at 222.5 mμ were: after 1 day, 0.588; 3 days, 0.546; 4, 0.553; 5, 0.559. Excess periodate was destroyed with ethylene glycol and the periodate-oxidised material reduced, without isolation, with excess potassium borohydride (50 mg.) for 48 hr. at room temperature. Residual borohydride was destroyed with dilute acetic acid, the solution being made to about pH 5 or 6 (pH paper). The solution was deionised on a mixed bed ion-exchange resin column (10 ml. each of Amberlite I.R. 120 (H) and 45 (OH) and repeatedly

evaporated with methanol to remove borate ions. The reduced product was hydrolysed (N sulphuric acid, room temperature, 3 hr.) and chromatography of the hydrolysate (10/3/3) showed two spots, one with the mobility of xylose and one with the mobility of glycerol. Both spots were non-reducing, not being shown up with the aniline phthalate spray. Xylose and glycerol were demonstrated in a complete acid hydrolysis of the mild acid hydrolysate. The non-reducing component of the mild acid hydrolysate with R_x 1.0 was probably xylosyl-glycerol although this has not been confirmed. The identification of xylose in the complete acid hydrolysate indicates that xylose substituted in position 3 was present in the oligosaccharide fraction. Assuming that all the acetal links in the poly-alcohol have been broken by the mild acid hydrolysis this periodate resistant xylose must form part of rhodymenabiose fragments present as part of the structure of the oligosaccharide mixture.

The presence of xylose units substituted in position 3 was confirmed by the chromatographic identification of 2:4 di-O-methyl xylose after hydrolysis of the product of methylation of the remainder of the oligosaccharide fraction. R_f 0.40 (0.42 quoted above) in 200/17/1. Tri-O-methyl xylose 0.77 (0.78) was also identified.

Discussion

Owing to the large amount of periodate and resulting inorganic material necessarily present in the initial oxidation it was decided that the poly-aldehyde would have to be isolated, free from inorganic material, before reduction to the poly-alcohol.

The low yields obtained by the dialysis procedure prompted the use of ion-exchange resins as a means of conserving the carbohydrate material. In point of fact the yield obtained by this procedure was less than that obtained by dialysis.

It is apparent that, in view of the preponderance of xylose and xylitol, both as free sugars and as components of the various fractions isolated, and the relative scarcity of glycerol - especially noticeable in the complete absence of free glycerol in the low molecular weight fraction - the yield of 4.4 g. from 20 g. of xylan is not a representative 22% yield. It probably represents a higher recovery of the periodate resistant xylose units at the expense of the fragments representing the oxidised xylose residues. Taking as an average the ratio of 1-4/1-3 linked xylose units to be 75/25% quoted above, the theoretical yield of periodate resistant xylose is 5 g. It may be estimated that substantially more than 50% of this was recovered in the 4.4 g. total yield.

The presence of xylitol in both the low molecular weight and poly-alcohol fractions shows that the poly-aldehyde fraction was fragmented during the deionisation procedure exposing free xylose and xylose end-groups to subsequent reduction. It is evident that the poly-aldehyde must have been intact up to this point as xylose end-groups exposed by chain breakdown would have been immediately oxidised by the periodate.

The large quantities of free xylose present in the low molecular weight fraction can only have come from hydrolysis of the reduced product after the destruction of the potassium borohydride. This hydrolysis must have taken place on the I.R. 120 (H) column. This points in turn to the probability of the fragmentation of the poly-aldehyde having taken place on the I.R. 120 column during the deionisation procedure.

This apparent acid lability of both poly-aldehyde and poly-alcohol was evident again in attempts to establish mild acid hydrolysis conditions for the breakdown of the poly-alcohol. Free xylose was liberated, even under the mildest conditions used. The conditions eventually employed were those used by Aspinall and Ross (1963) under which xylosyl-glycerol was reported to be stable.

The separation of the hydrolysis products provided a number of problems in that compounds chromatographically homogeneous were separated into multiple components on electrophoresis. The isolation of the unidentified compound Z and the failure to isolate substantial quantities of xylosyl-glycerol and rhodymenabiosyl-glycerol points to a marked alteration in the theoretical pathway set out in Fig. 10. However, the isolation and characterisation of rhodymenabiose, the presence of rhodymenabiose units in the 'trisaccharide' LMW 1 and the probable presence of rhodymenabiose fragments in the oligosaccharide fraction shows conclusively that there are a small number of adjacent β, 1-3 linkages in the xylan.

It is of interest to note the similarity between LMW 1 and compound W. They are both chromatographically homogeneous, and are both split into three components on electrophoresis with M_x values respectively of 0.46, 0.69 and 0.82 and 0.43, 0.63 and 0.84. On hydrolysis they both yield xylose and compound Z. It may not be out of place to suggest that, in view of the presence of rhodymenabiose units in LMW 1, this 'trisaccharide' contains homologues of the components of compound W each containing one more xylose residue.

The isolation of the oligosaccharide fraction, containing mainly xylose units as demonstrated by hydrolysis, could possibly have yielded

information on the probability of blocks of 1-3 linkages but in view of the difficulty of hydrolysis of the poly-alcohol (viz. three hydrolyses in N. sulphuric acid at room temperature to effect approximately total fragmentation) and the probable complexities in the analysis of the 40 mg. available only the experiments reported above were undertaken.

As a result of the work reported it may be concluded that there exists in the xylan molecule a few adjacent β, 1-3 linkages. The presence of large amounts of xylose in the hydrolysate of the poly-alcohol shows however that in the main, the 1-3 linkages are singly distributed throughout the molecule. This indicates a random rather than a highly ordered structure for the xylan from Rhodymenia palmata.

Section III

The enzymic degradation of Rhodymenia palmata xylan.

Enzymic hydrolysis as a tool for the structural examination of polysaccharides is advantageous in that the hydrolysis is much more easily controlled than in acid hydrolysis. The random splitting of polysaccharides by enzyme action and the examination of the fragments produced yields useful information of the molecular structure. An obvious example of this is the use of amylases in the structural examination of starch-type polysaccharides. Another example is that reported by Howard (1957) who used a non-specific preparation from a mixed bacterial culture to hydrolyse the xylans from wheat flour and Rhodymenia palmata.

The technique of enzymic hydrolysis may be refined by the use of enzymes specific for one type of glycosidic linkage in structural investigations of polysaccharides with more than one type of linkage in the molecule. This method has been applied to oat and barley glucans, containing both β, 1-4 and β, 1-3 linked glucose residues, by Parrish et al (1960) and to lichenin, a similar glucan from Iceland moss, by Cunningham and Manners (1961) using fungal enzyme preparations.

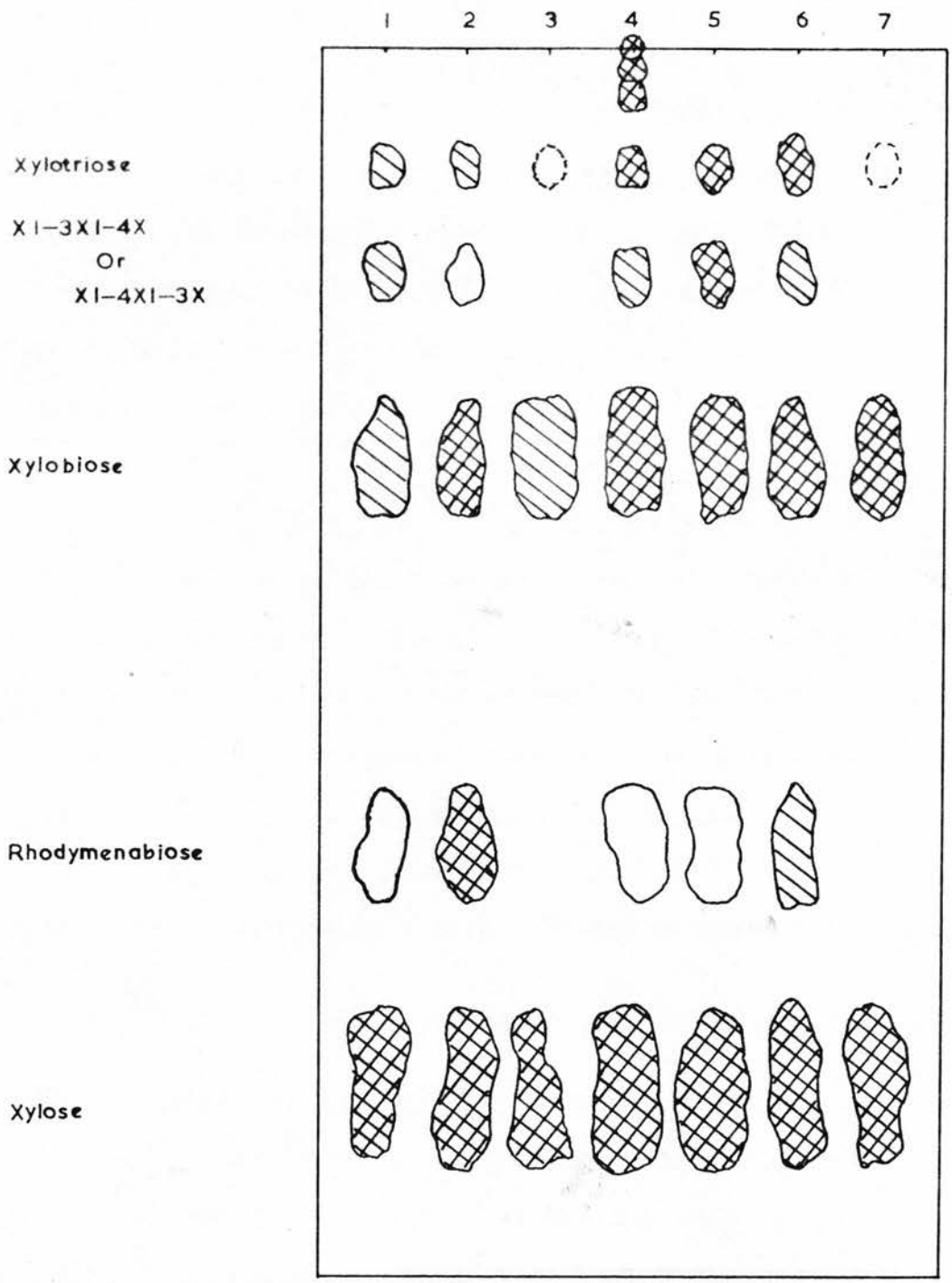
Bishop (1956) reported that an enzyme specifically hydrolysing β, 1-4 linked xylose residues was present in a preparation from the mould, Myrothecium verrucaria and Bishop and Whitaker (1955) report the use of this enzyme in the hydrolysis of a wheat straw xylan, isolating xylose/arabinose oligosaccharides which were unobtainable by conventional acid hydrolyses because of the acid lability of the arabinofuranoside linkages.

In view of this apparent specificity of the Myrothecium xylanase a preparation from a culture filtrate was made and tested (Methods and Materials) for use in conjunction with extracts from Rhodymenia and Cladophora in the investigations reported in this section.

Experimental

As a preliminary to enzymic hydrolyses of xylan samples, qualitative digests were prepared with extracts of Cladophora rupestris (C), Rhodymenia palmata (P) and Myrothecium verrucaria in order to test the extracts for xylose transferase activity. Xylobiose and p-nitrophenyl β-D-xyloside were used as substrates in digests incubated at pH 6.0 at 37°C. After one week, xylotriose and a reducing sugar with the same mobility as the trisaccharide $3^2\beta$- xylosyl-xylobiose were detected on

Xylo-transferase activity



Control digests after 4 weeks incubation:

1, Cladophora; 2, Rhodymenia, 3, Myrothecium extracts with p-nitrophenyl-ß-D-xyloside.

4, Rhodymenia xylan, partial acid hydrolysate.

5, Cladophora, 6, Rhodymenia, 7, Myrothecium extracts with xylobiose.

FIGURE 12

chromatographic examination (in 10/3/3) of the xylobiose digests with Cladophora and Rhodymenia extracts. The same two sugars, and xylobiose were detected in the p-nitrophenyl xyloside digests with these extracts. Rhodymenabiose was also detected in the Cladophora and Rhodymenia digests, probably arising from direct synthesis in the p-nitrophenyl xyloside digests, but more likely from synthesis and subsequent hydrolysis of the trisaccharide containing both a 1-4 and a 1-3 linkage, in the xylobiose digests. With the Myrothecium extract, xylobiose and a trace of xylotriose were detected in the p-nitrophenyl xyloside digest, and a trace of xylotriose in the xylobiose digest. After four weeks, all the synthetic products were observed in increased yield. (Fig. 12). In view of the considerable synthesis of β, 1-3 linkages observed in the Cladophora and Rhodymenia digests, only the Myrothecium preparation was of any practical use in enzymic examination of the xylan structure with a view to isolating xylo-oligosaccharides linked β, 1-3.

Enzymic hydrolysis of Rhodymenia xylan by a Myrothecium verrucaria preparation.

Xylan M. was incubated (1 g. 2% solution pH 6.0 37°C.) with Myrothecium preparation (2 g.). After four weeks the digest was deproteinised (zinc sulphate/barium hydroxide) and unhydrolysed polysaccharide (approx. 50 mg.) precipitated from 50% acetone. The concentrated oligosaccharide mixture was chromatographed on a column of Sephadex G-25 (110 x 2.5 cm. 540 ml.). 5 ml. fractions were collected and examined chromatographically in 10/3/3. Those fractions containing the trisaccharide material and an overlap into the tetra and di-saccharide containing fractions were combined and put on to the charcoal/Celite column

used in the separation of the hydrolysate of the poly-alcohol above. Those fractions containing mainly xylose and a small amount of xylobiose (total 400 mg.) and those containing the higher oligosaccharides (350 mg.) were not examined further.

The column was washed with about a litre of water and of 2% ethanol. An 8% ethanol eluate was collected in 25 ml. fractions but not until about 40 fractions had been collected was a positive reaction obtained with the phenol/sulphuric acid reagent. The eluting mixture was changed to 12% ethanol and 34 fractions collected, the last of which gave negligible results with the phenol/sulphuric acid reagent. The concentrated eluate was shown chromatographically and electrophoretically to contain xylobiose and a trace of rhodymenabiose. Separation on thick paper (in 10/3/3) yielded 40 mg. of pure xylobiose as a hygroscopic solid with $(\alpha)_D$ -25.8° in water; Whistler and Tu (1951) record -25.5°; Howard (1957) records -25°.

30 fractions of an 18% ethanol eluate were collected. Electrophoresis and chromatography showed this to contain mainly rhodymenabiose and a trace of xylobiose. These two components were separated on thick paper (in 10/3/3) yielding about 10 mg. rhodymenabiose R_x 0.76 (0.77) in 10/3/3 and M_x 0.60 (0.60). The xylobiose present only a trace gave R_x 0.69 (0.71) in 6/4/3. This solvent was used to distinguish xylobiose from possible contamination with rhodymenatriose which has the same chromatographic mobility as xylobiose in 10/3/3.

The column was next eluted with 25% ethanol and thick paper separation of the concentrated eluate yielded three bands (in 10/3/3), fractions 25/1, /2 and /3. The least mobile of these, fraction 25/1, was isolated as 50 mg. of hygroscopic solid chromatographically and electrophoretically indistinguishable from authentic xylotriose with $(\alpha)_D$ -44 in water; Whistler and Tu (1951) -47°, Howard (1957) -45° and Bishop (1955) -44.4°.

Fraction 25/2 was eluted and shown to be split into two components on electrophoresis. These two components were separated electrophoretically (2 papers, Whatman No. 3MM. 22 x 7.5 cm. 250 V. 2.5 hr.). The two bands I and II were eluted, freed from sodium on a small I.R. 120 (H) column and from boric acid by repeated evaporation with methanol, and examined chromatographically and electrophoretically. R_x I, 0.56; II, 0.61 (in 6/4/3); I, 0.27; II, 0.31 (in 10/3/3) M_x I, 0.14; II, 0.46.

Fractions I and II were dissolved in 100 and 50 ml. water respectively and the xylose content determined by the phenol/sulphuric acid method. Mean extinctions obtained were I, 0.908, II, 0.170, showing the amounts of I and II to be about 5.3 and 0.5 mg. respectively. Small samples (5 ml.) of these solutions were reduced with excess potassium borohydride for 60 hr. and the xylose content again determined by the phenol/sulphuric acid method. Extinctions: I, 0.540, II, 0.120. These figures give values for the D.P. of 2.6 and 3.3 respectively, showing both fractions to be trisaccharides.

The remainder of the solutions of unreduced I and II were concentrated and reduced with excess potassium borohydride for three days. After destruction of residual borohydride with dilute acetic acid,

de-ionising on a mixed-bed resin column of I.R. 120 (H) and 45 (OH) and treatment with methanol to remove borate, partial acid hydrolysis (0.1 N sulphuric acid, 30 min. 98°C.) and chromatography in 10/3/3 yielded rhodymenabiose and xylose from I and traces of xylobiose and xylose from II. The chromatogram was sprayed with aniline phthalate to avoid developing non-reducing sugar spots with unknown mobilities.

These partial acid hydrolysis results show that I must be $3^2\beta$ - xylosyl xylobiose and II, $4^2\beta$ - xylosyl rhodymenabiose. These conclusions are supported by the results of electrophoresis in which compounds containing 1-3 linked reducing end-groups are more mobile than those with 1-4 linked end-groups (Foster, 1953; Howard, 1957).

Fraction 25/3 was present only in trace quantities but concentration and chromatographic separation on Whatman No. 1 paper in 6/4/3 gave two components. The major component was found to be indistinguishable from xylobiose, R_x 0.39 (0.40) in 10/3/3; 0.69 (0.70) in 6/4/3 and M_x 0.2 (0.2). The minor component gave R_x 0.78 in 6/4/3 and 0.13 in a 10/3/3 tank in which standard oligosaccharides ran much slower than expected. Run on the same papers as this minor component, standard rhodymenatriose from a Gaulerpa filiformis xylan partial acid hydrolysate, (Methods and Materials) gave R_x 0.78 and 0.13. Electrophoretically this minor component gave an elongated spot with $M_x > 0.5$.

Gas chromatographic analysis of the methanolysed product of a methylation of the remainder of the minor component showed the presence of the methyl glycoside of 2:4 di-O-methyl xylose as a major component, confirming the presence of xylose units substituted at C_3 in the oligosaccharide.

The evidence obtained from chromatography and electrophoresis and from methylation studies indicated that the minor component was, in fact, rhodymenatriose.

The column was finally eluted with 30 and 40% ethanol (1000 ml. of each) yielding two further components. One was indistinguishable from xylotetraose (10 mg.) R_x 0.04 (0.04) in 10/3/3; M_x 0.14 (0.14); $(\alpha)_D$ $-60^\circ \pm 2^\circ$, Whistler and Tu (1951) quote -60° for xylotetraose. The other component (5 mg.) had R_x 0.08 in 10/3/3 and M_x 0.14, this latter figure indicating that this sugar probably contains a 1-4 linked reducing end-group. Howard (1957) reported R_x 0.08 in 10/3/3 for a tetrasaccharide with the probable structure X1-4X1-3X1-4X (where X = xylose) which is probably the same as this second component although this was not examined further.

Discussion

The oligosaccharides obtained in the charcoal chromatographic fractions examined after enzymic hydrolysis of Rhodymenia palmata xylan are shown in the table below.

Eluant	Oligosaccharide	Yield
12% ethanol	xylobiose	40 mg.
	rhodymenabiose	trace
18% ethanol	rhodymenabiose	10 mg.
	xylobiose	trace
25% ethanol	xylotriose	50 mg.
	X1-3X1-4X	5 mg.
	X1-4X1-3X	0.5 mg.
	rhodymenatriose	<1 mg.
30/40% ethanol	xylotetraose	10 mg.
	X1-4X1-3X1-4X(?)	5 mg.

All of these oligosaccharides, with the exception of rhodymenatriose, were obtained by Howard (1957). Although he did not specifically identify the trisaccharide $4^2\beta$ - xylosyl rhodymenabiose (Xl-4Xl-3X), he reported the presence of a compound with the same chromatographic mobility as Xl-3Xl-4X but with M_x 0.5 (in 0.2 M. borate buffer pH 10) which must in fact have been this compound Xl-4Xl-3X.

The isolation of rhodymenatriose in very small yield demonstrates the presence of a small number of adjacent 1-3 linked xylose units in the xylan molecule thus confirming the similar result obtained by the 'Smith degradation' procedure described in Section II of this chapter.

Summary

In the light of the work reported in this chapter the picture presented of Rhodymenia palmata xylan is one of a molecule of about 100 xylose units, depending on the method of isolation, with a possible slight degree of branching. Within the molecule, the xylose residues are linked β, 1-4 and β, 1-3 in the ratio 75/25% and in the main the β, 1-3 linked xylose residues are singly placed throughout the molecule although a small number occupy adjacent positions.

It is regretable that in view of the weak xylanase activity of the available extracts of Cladophora and Rhodymenia and their contamination with xylose transferase activity further investigation of the enzymic degradation of this xylan by algal enzymes was impracticable.

Chapter III

STUDIES ON THE β-GLYCOSIDASE ACTIVITIES OF CLADOPHORA RUPESTRIS, RHODYMENIA PALMATA AND LAMINARIA CLOUSTONI.

β-Glycosidase enzymes catalyse the hydrolysis of the β-glycosidic linkage between either two sugar units (e.g. cellobiose or xylobiose) or between a sugar unit and an aglycone moiety (e.g. phenyl-β-D-glucoside or p-nitrophenyl-β-D-xyloside). A β-glycosidase may also catalyse the stepwise degradation of a polysaccharide from the non-reducing end. These enzymes occur widely in Nature, having been reported in a variety of higher plants, mammalian tissues and in many moulds and bacteria (Veibel, 1950).

The apparent specificity of β-glycosidases varies with the source. The β-glucosidase and β-galactosidase of pig intestinal mucosa have been shown by studying the effect of pH, partial deactivation and inhibitors to be indistinguishable (Dahlqvist, 1961; Heyworth and Dahlqvist, 1962), while the fungus Stachybotrys atra contains readily separable β-glucosidase enzymes, one of which will act only on aryl-β-D-glucosides, another which will catalyse the hydrolysis of aryl-β-D-glucosides and -cellobiosides and a third active towards neither of these substrates but acting on cellotriose and higher cello-oligosaccharides, (Jermyn, 1961). Murti and Stone (1961) separated the β-glucosidases of the mould Aspergillus niger by column chromatography into oligosaccharase and polysaccharase fractions acting respectively on cello-oligosaccharides and cellodextrins as substrates.

Miwa and Tanaka (1949) demonstrated independent variation in relative β-glucosidase, β-galactosidase and β-xylosidase activities during fractionation and inhibition studies on apricot emulsin. From the same source Baba (1957) reported the separation of a fraction rich in β-xylosidase activity and other fractions containing approximately equal β-glucosidase and β-xylosidase activities, while Nishizawa et al (1961) report the separation of β-glucosidase, β-galactosidase and β-xylosidase activities by electrophoresis of an apricot emulsin preparation.

Morita (1952) obtained the β-glucosidase from taka-diastase (Aspergillus oryzae preparation) free from β-xylosidase activity, but was unable to effect the reverse purification. He concluded that these two activities were due to separate enzymes.

The position of sweet almond emulsin β-glycosidase specificity is still in some doubt. Helferich and his co-workers (for a review, see Veibel, 1950) were unable, by extended fractionation and partial deactivation experiments, to show any variation in the ratios of the various β-glycosidase activities. Heyworth and Walker (1962) studying the hydrolytic properties and the effect of pH, partial deactivation and inhibitors concluded that the β-glucosidase and β-galactosidase activities were due to one enzyme. On the other hand, Helferich and Jung (1958) reported the separation of these two activities on columns of p-hydroxy-styrene glycoside polymers. By this technique substantial variation in the ratios of the two activities was achieved, but Helferich and Kleinschmidt (1961) were unable, by a prolonged purification procedure using the classical techniques of acetone and ammonium sulphate precipitation, to show any significant variation in the ratio of the two activities in the

crude extract and in the highly purified preparation. Wagner and Pflegel (1962) using thio-phenyl O,S di-glycosides as enzyme substrates concluded that the β-glucosidase and β-galactosidase of almond emulsin were due to different enzymes.

β-Glycosidase activity towards a wide range of substrates has been reported by Duncan et al (1956) in extracts of the marine algae Cladophora rupestris, Ulva lactuca, Rhodymenia palmata and Laminaria digitata while Peat and Rees (1961) demonstrated β-glucosidase and β-galactosidase activity in extracts of Porphyra umbilicalis.

In the work described in this chapter extracts of Cladophora rupestris, Rhodymenia palmata and Laminaria clouatoni were used to examine the β-glucosidase, β-galactosidase and β-xylosidase activities to determine whether these activities were readily distinguishable. A sweet almond emulsin preparation was used as a standard for comparison.

Experimental

Enzymic activity was assayed by the procedures outlined in Methods and Materials. Unless otherwise stated, the substrate solutions used were 0.001 M p-nitrophenyl β-D-glycosides. The Cladophora and Rhodymenia extracts used were C and P respectively.

In order to confirm the validity of the p-nitro phenol assay technique for measuring enzymic activity, digests were prepared containing extract solution (2 ml. almond emulsin solution, various concentrations), substrate solution (5 ml. 0.001 M nitrophenyl β-D-glucoside), B.D.H. Universal buffer pH 5.6 (2 ml.) and water (1 ml.) and incubated at 37°C. for 45 min. Samples (1 ml.) added to B.D.H. Universal buffer pH 9.8 (5 ml.) gave solutions which had the following extinctions at 400 mμ.

Extract solution mg. emulsin/ml.	0.025	0.050	0.075	0.100	0.150
Extinction	0.158	0.293	0.416	0.552	0.816

These results show a linear relationship and confirm that the p-nitro phenol released is proportional to the enzyme concentration, making its estimation a valid measure of enzymic activity

Determination of the ratio of β-glucosidase, β-xylosidase and β-galactosidase in the three algal extracts.

Digests were prepared containing 4 ml. extract solution, 4 ml. substrate solution and 2 ml. B.D.H. Universal buffer pH 5.2 and incubated at 36°C. The extract solutions contained 10 mg. extract/ml. (Rhodymenia) and 20 mg./ml. (Cladophora and Laminaria) 1 Ml. samples were taken at intervals and the liberated p-nitro-phenol determined by measuring extinctions at 400 mμ.

Extinction results

Time (days)	1	2	3	5	7	10
Cladophora						
Glucosidase	0.592	0.808	1.03	1.21	1.26	1.33
Xylosidase	0.362	0.446	0.574	0.713	0.850	1.02
Galactosidase	0.290	0.298	0.329	0.376	0.422	0.482
Rhodymenia						
Glucosidase	0.421	0.554	0.683	0.827	0.932	1.025
Xylosidase	0.218	0.276	0.340	0.398	0.452	0.509
Galactosidase	0.508	0.740	0.962	1.11	1.16	1.22

Time (days)	1	4	7	10	32
Laminaria					
Glucosidase	0.159	0.359	0.500	0.610	1.02
Xylosidase	0.110	0.216	0.293	0.356	0.623
Galactosidase	0.102	0.093	0.116	0.118	0.117

Ratio of glycosidase activities

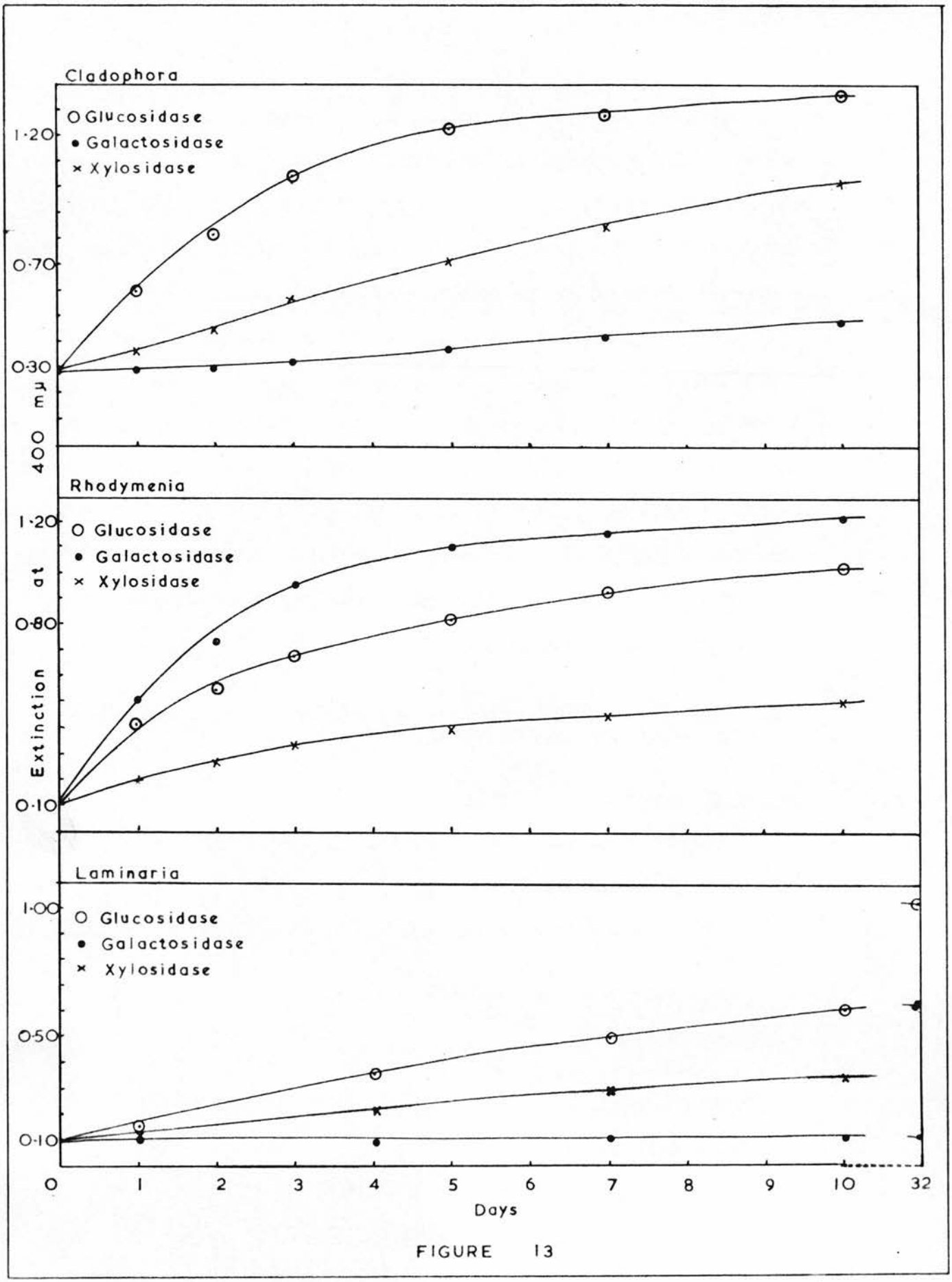


FIGURE 13

The above extinctions are inclusive of reagent and enzyme blank values. Initial values for the enzyme control were obtained by plotting the results (Fig. 13) and extrapolating to zero time. This gave E_{400} values of 0.270 for Cladophora, 0.100 for Rhodymenia and 0.09 for Laminaria. A net extinction of 1.1 represents 100% hydrolysis.

The ratio glucosidase/xylosidase/galactosidase calculated from the 3 day results was 12.9/5.2/1 for Cladophora and 0.72/0.28/1 for Rhodymenia. In the Laminaria extract the galactosidase was so weak that no valid use can be made of the results. This activity was not examined further in the experiments described below. The ratio glucosidase/xylosidase from the 10 day results was 2.0/1. These figures may be compared with that of about 40/1 for the ratio glucosidase/xylosidase in the almond emulsin preparation examined below.

Examination of the ratio glucosidase/xylosidase in various fractions of Cladophora and almond preparations.

Almond preparations

A number of fractions were prepared as listed below.

1. Crude de-fatted almond powder.
2. Residue after ethanol extraction of 1. (See Methods and Materials)

An aqueous extract of 2. fractionated with ammonium sulphate gave the next three fractions.

3. 0-35% saturation.
4. 36-42% saturation.
5. 43-70% saturation.
6. Emulsin (See Methods and Materials).

Digests were prepared containing 10 ml. substrate solution, 5 ml. B.D.H. Universal buffer pH 5.1 and 2 ml. extract solution and incubated at 35°C. Liberated p-nitro-phenol was estimated in samples taken at intervals.

Fraction 6 (emulsin) in a solution containing 1 mg./ml. added to the above digest gave the following results (Extinctions at 400 mμ.).

Time (min.)	10	30
Glucosidase	0.633	1.159
Xylosidase	0.026	0.053
Glucosidase/Xylosidase	39	27

Fraction 4 (2 mg./ml.) gave the following results.

Time (min.)	30	60
Glucosidase	0.588	0.856
Xylosidase	0.036	0.044
Glucosidase/Xylosidase	22	25

This method of direct comparison was discontinued because of the very low extinction values from the xylosidase digests. Much stronger extract solutions were prepared and used for the xylosidase digests and after accurate dilution, the same solutions were used for the glucosidase activity measurements. The initial concentration and the dilution factor for the various fractions are listed below.

1. 1 G./25 ml. diluted 26-fold.
2. 2 G./25 ml. diluted 26-fold.
3. 400 Mg./5 ml. diluted 26-fold.
5. 20 Mg./10 ml. diluted 26-fold.
6. 100 Mg./10 ml. diluted 30-fold.

Digests of the composition outlined above were incubated for 30 min. at 35°C. and the glycosidase activity determined in the usual way. The results are given as extinction values at 400 mμ.

Fraction	Glucosidase activity	Xylosidase activity	Glucosidase/Xylosidase
1.	0.467;0.462	0.284;0.274	44
2.	0.560	0.310	47
3.	0.299	0.194	40
5.	0.383	0.237	42
6.	0.594	0.452	40

In calculating the ratios quoted above allowance is made for the dilutions, the glucosidase results being multiplied by the dilution factor before calculating the ratio.

Cladophora fractions

Cladophora extract C (5 g.) was stirred for 2 hr. at 0°C. with 500 ml. water. After the large insoluble residue had been removed, protein material was precipitated from the solution by successive additions of ammonium sulphate to 18, 36, 54 and 72% saturation. After each addition of ammonium sulphate the solution was kept at 0°C. for 30 min. and the precipitated protein separated by centrifugation, dissolved in a little water and dialysed free of sulphate.

Fractions W, X, Y and Z amounting to 47, 215, 139 and 74 mg. respectively, were obtained by freeze-drying the solutions.

The β-glucosidase and β-xylosidase of fractions X, Y and Z were examined in digests containing 2 ml. extract solution (5 mg./ml.), 2 ml. substrate solution and 1 ml. B.D.H. Universal buffer pH 5.8 incubated at 35°C. for 22 hr.

Fraction	X	Y	Z
Extinctions			
Glucosidase	0.418	0.726	0.148
Xylosidase	0.043	0.216	0.014
Glucosidase/Xylosidase	9.7	3.3	10.6

Although fraction Y is the most active in both glucosidase and xylosidase, the wide variation in the ratio of the two activities in the different fractions suggests that these two activities in the Gladophora extract are due to separate enzymes. This wide variation contrasts with the small variation of doubtful significance in the ratio of these activities in the almond preparations.

Investigation of the effect of pH on the glycosidase activities of the algal extracts and almond emulsin.

Emulsin

Digests containing substrate solution (10 ml.), B.D.H. Universal buffer, (5 ml.), water (3 ml.) and enzyme solution (2 ml.) were prepared and incubated at 35°C. for 45 min. The activities were determined in the usual way. Enzyme solutions contained 10 mg. emulsin/35 ml. water for the glucosidase assay and 250 mg./25 ml. for the xylosidase assay.

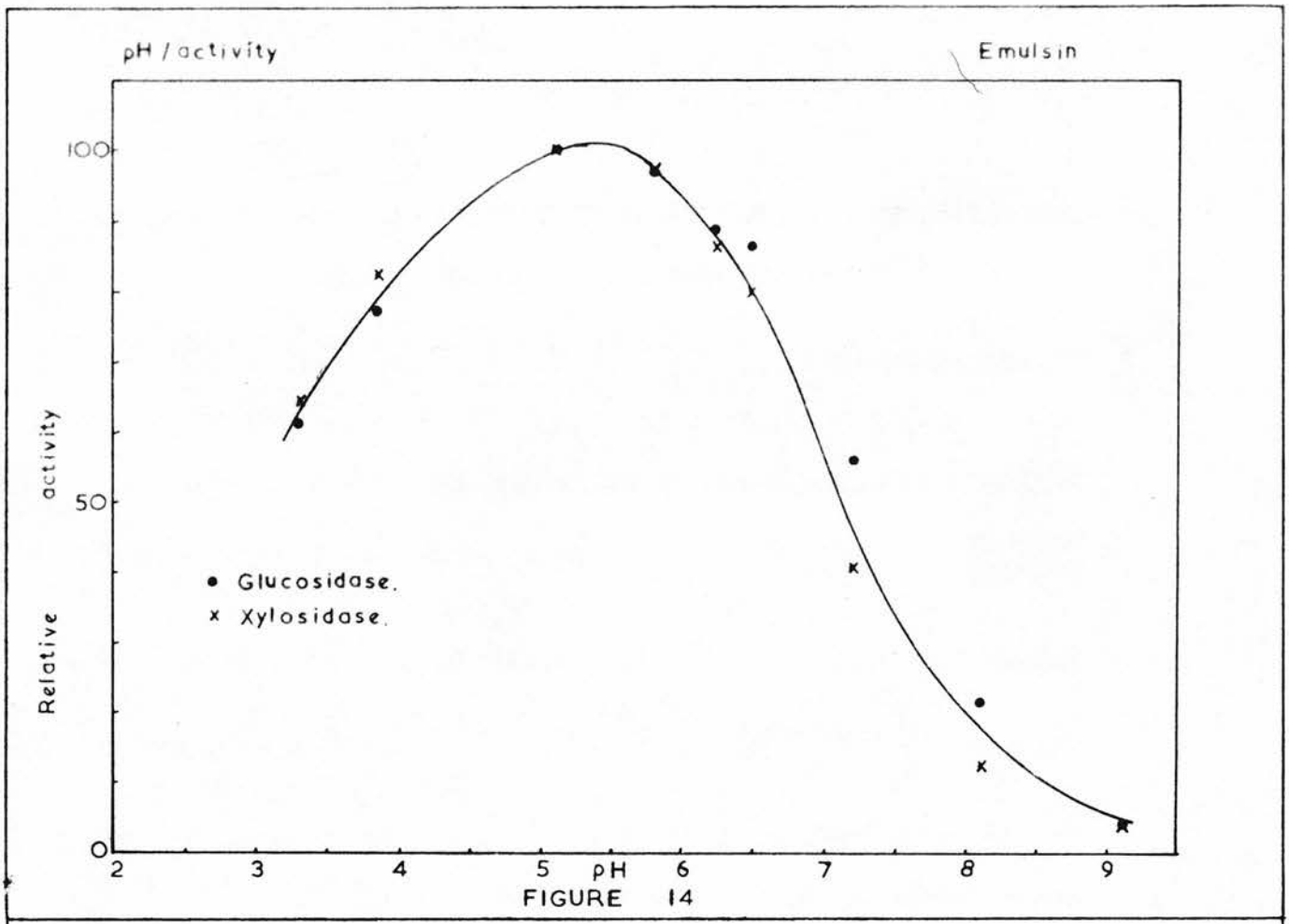


FIGURE 14

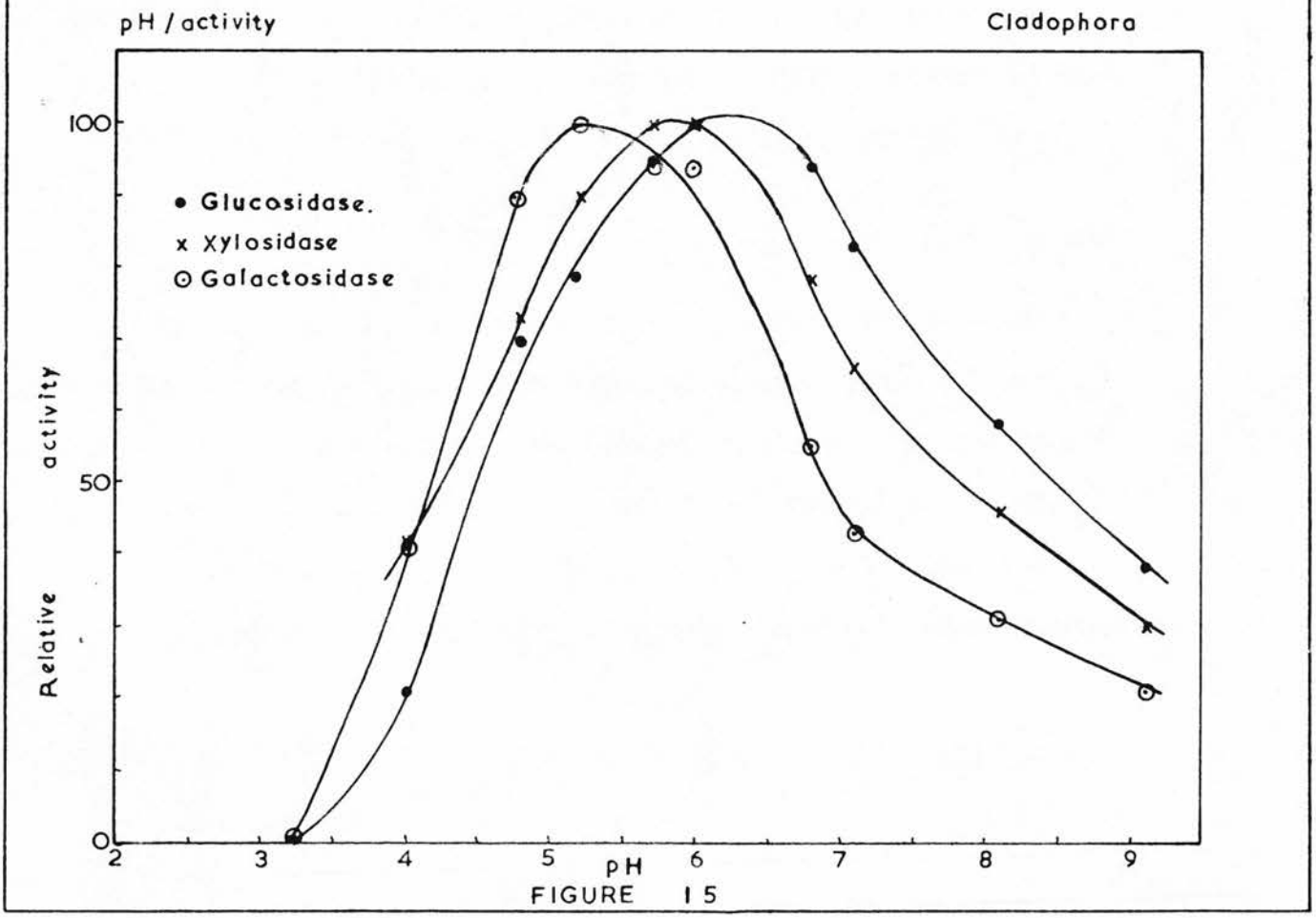


FIGURE 15

Direct comparison of the two activities was achieved by making the highest extinction equivalent to 100 in both cases.

Extinction values

pH	3.3	4.1	5.1	5.8	6.2	6.5	7.2	8.1	9.2
Glucosidase	0.410	0.530	0.685	0.663	0.609	0.592	0.384	0.148	0.029
Relative activity	61.3	77.4	100	97.0	89.0	86.7	56.0	21.6	4.2
Xylosidase	0.367	0.468	0.569	0.557	0.491	0.455	0.230	0.070	0.020
Relative activity	64.4	82.3	100	97.5	86.2	79.9	40.4	12.3	3.5

In both cases the optimum pH is 5.5. A plot of these results (Fig. 14) gives practically the same curve in each instance.

In the examination of the algal extracts a standard digest containing 2 ml. extract solution, 2 ml. substrate solution and 1 ml. B.D.H. Universal buffer was used.

Cladophora

Extract solutions were prepared containing 10 mg./ml. for the glucosidase and galactosidase assays and 20 mg./ml. for the xylosidase assay. Glucosidase and xylosidase digests were incubated for 2 days and the galactosidase digests for 4 days, all at 36°C. Liberated p-nitro-phenol was estimated in the usual way. The results are quoted as extinctions at 400 mμ.

Glucosidase

pH	3.2	4.0	4.8	5.2	5.7	6.0	6.8	7.1	8.1	9.1
	0.00	0.101	0.339	0.381	0.463	0.485	0.457	0.403	0.281	0.186
Relative activity	0	21	70	79	95	100	94	83	58	38

Xylosidase

pH	4.0	4.8	5.2	5.7	6.0	6.8	7.1	8.1	9.1
	0.133	0.230	0.286	0.316	0.317	0.246	0.208	0.145	0.096
Relative activity	42	73	90	100	100	78	66	46	30

Galactosidase

pH	3.2	4.0	4.8	5.2	5.7	6.0	6.8	7.1	8.1	9.1
	0.00	0.033	0.072	0.080	0.075	0.075	0.044	0.034	0.025	0.017
Relative activity	0	41	90	100	94	94	55	43	31	21

Plotting the above results (Fig. 15) it is apparent that the glucosidase, xylosidase and galactosidase activities have distinct pH/activity curves with maximum activity at pH's 6.3, 5.9 and 5.2 respectively. This supports the suggestion that each glycoside is hydrolysed by a different enzyme in the Cladophora extract.

Rhodymenia

The extract solutions for the galactosidase and xylosidase digests contained 10 mg./ml. and that for the glucosidase digest 5 mg./ml. Xylosidase and glucosidase digests were incubated for 24 hr. and the galactosidase digests for 48 hr., all at 36°C. The p-nitro-phenol liberated in these digests was determined in the usual way and the results quoted as extinction values at 400 mμ.

Glucosidase

pH	4.0	4.8	5.2	5.8	6.0	6.8	7.1	8.1	9.1
	0.111	0.194	0.249	0.294	0.296	0.237	0.196	0.135	0.042
Relative activity	38	66	84	100	100	80	66	46	14

Xylosidase

pH	3.2	4.0	4.8	5.2	5.8	6.0	6.8	7.1	8.1	9.1
	0.060	0.094	0.182	0.204	0.237	0.239	0.178	0.123	0.095	0.90
Relative activity	25	40	76	86	100	100	75	52	40	-

Galactosidase

pH	4.0	4.8	5.2	5.8	6.0	6.8	7.1	8.1	9.1
	0.613	0.520	0.483	0.456	0.420	0.198	0.121	0.030	0.00
Relative activity	100	85	79	74	69	33	20	5	0

A subsequent examination of the effect of acid pH on the galactosidase activity gave the following results, quoted as extinctions at 400 mμ. after three days incubation.

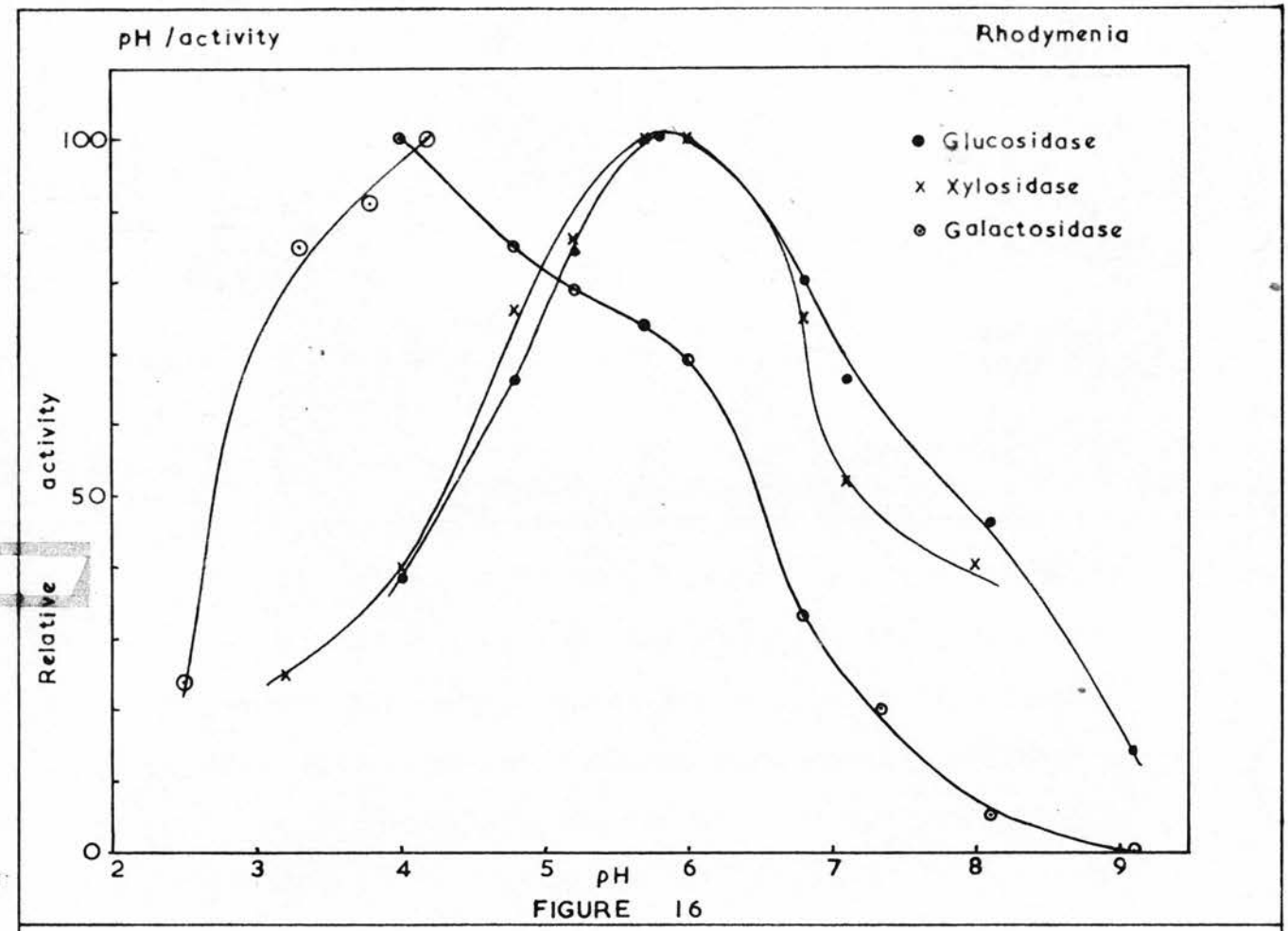


FIGURE 16

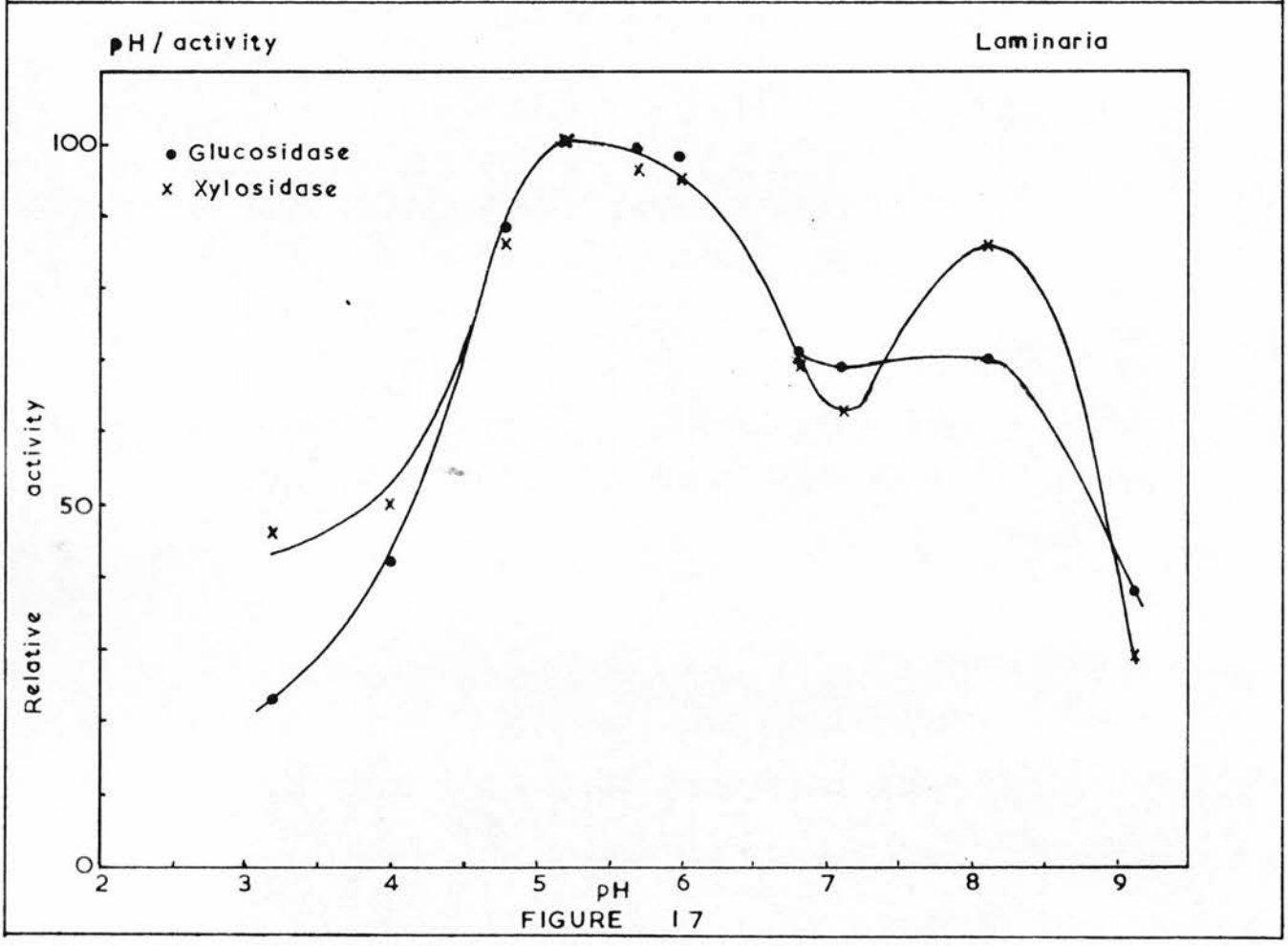


FIGURE 17

pH	2.6	3.3	3.8	4.2
	0.250	0.878	0.935	1.03
Relative activity	24	85	91	100

Plotting these results (Fig. 16) it is apparent that the glucosidase and xylosidase activities have similar pH/activity curves with maximum activity at pH 5.8. The galactosidase has an entirely different curve with maximum activity at pH 4.0-4.2. This indicates that the Rhodymenia extract contains at least two separate glycosidase enzymes, one hydrolysing glucosidic and xylosidic linkages and the other hydrolysing galactosidic linkages. An examination of the β-galactosidase pH/activity curve in Fig. 16 suggests the possibility of a second β-galactosidase with optimum activity between pH 5.5 and 6.0.

Laminaria

Extract solutions contained 10 mg./ml. and the digests were incubated for 3 days at 36°C. Extinction results are given below.

Glucosidase

pH	3.2	4.0	4.8	5.2	5.7	6.0	6.8	7.1	8.1	9.1
	0.113	0.209	0.439	0.502	0.497	0.489	0.355	0.344	0.348	0.190
Relative activity	23	42	88	100	99	98	71	69	70	38

Xylosidase

pH	3.2	4.0	4.8	5.2	5.7	6.0	6.8	7.1	8.1	9.1
	0.135	0.149	0.263	0.296	0.285	0.282	0.204	0.185	0.255	0.086
Relative activity	46	50	86	100	96	95	69	63	86	29

The pH/activity curves (Fig. 17) show two maxima. These results suggest the presence of one enzyme with optimum activity within the range pH 5 - 6 which hydrolyses both glucosidic and xylosidic linkages and at least one other with maximum activity at about pH 8. This latter appears to be more active toward xylosidic linkages than toward glucosidic linkages.

Examination of the effect of heat on the various glycosidase activities.

Aqueous solutions of emulsin and the three algal extracts were heated at various temperatures. Glucosidase, xylosidase and galactosidase activities were then assayed in the usual way using the p-nitrophenyl-glycosides as substrates.

Emulsin

Samples (5 ml.) of an emulsin solution (200 mg./20 ml. water) were heated at 60°C. and 50°C. for 20 min., cooled to room temperature and the glucosidase and xylosidase activities assayed by means of the 20 ml. digest used in the pH/activity examination of emulsin above. Buffer pH 5.7 was used and the digests incubated at 35°C. for 30 min. before measuring the liberated p-nitro-phenol in the usual way. Each stock solution (1 ml.) was diluted 26-fold for the glucosidase assay. Extinction results are quoted below.

Temperature	Glucosidase activity	Xylosidase activity	Glucosidase/Xylosidase
R.T.	0.680	0.444	40
50°	0.588	0.388	40
60°	0.396	0.261	40

As in previous experiments allowance is made for the dilution before calculating the ratio of activities. Both activities are decreased due to de-naturing of the enzyme protein at higher temperatures but the ratio of activities remains the same. This supports the suggestion that these two activities are due to the same or closely related enzymes.

Cladophora

Glucosidase and xylosidase.

An extract solution containing 20 mg./ml. was used. Samples (2 ml. and 1 ml.) were heated for 15 min. at the appropriate temperature and cooled to room temperature. The 2 ml. sample was used with 2 ml. p-nitrophenyl-xyloside solution and 1 ml. B.D.H. Universal buffer pH 6.0 to assay xylosidase activity and the 1 ml. sample, diluted with 1 ml. water used in a similar digest with p-nitrophenyl glucoside to assay the glucosidase activity. Extinction results are quoted below.

Temperature (°C.)	20	41	49	60	65	70
Glucosidase						
	0.508	0.480	0.440	0.358	0.238	0.085
Relative activity	100	95	87	71	47	17
Xylosidase						
	0.385	0.387	0.377	0.300	0.230	0.118
Relative activity	100	100	98	78	60	31

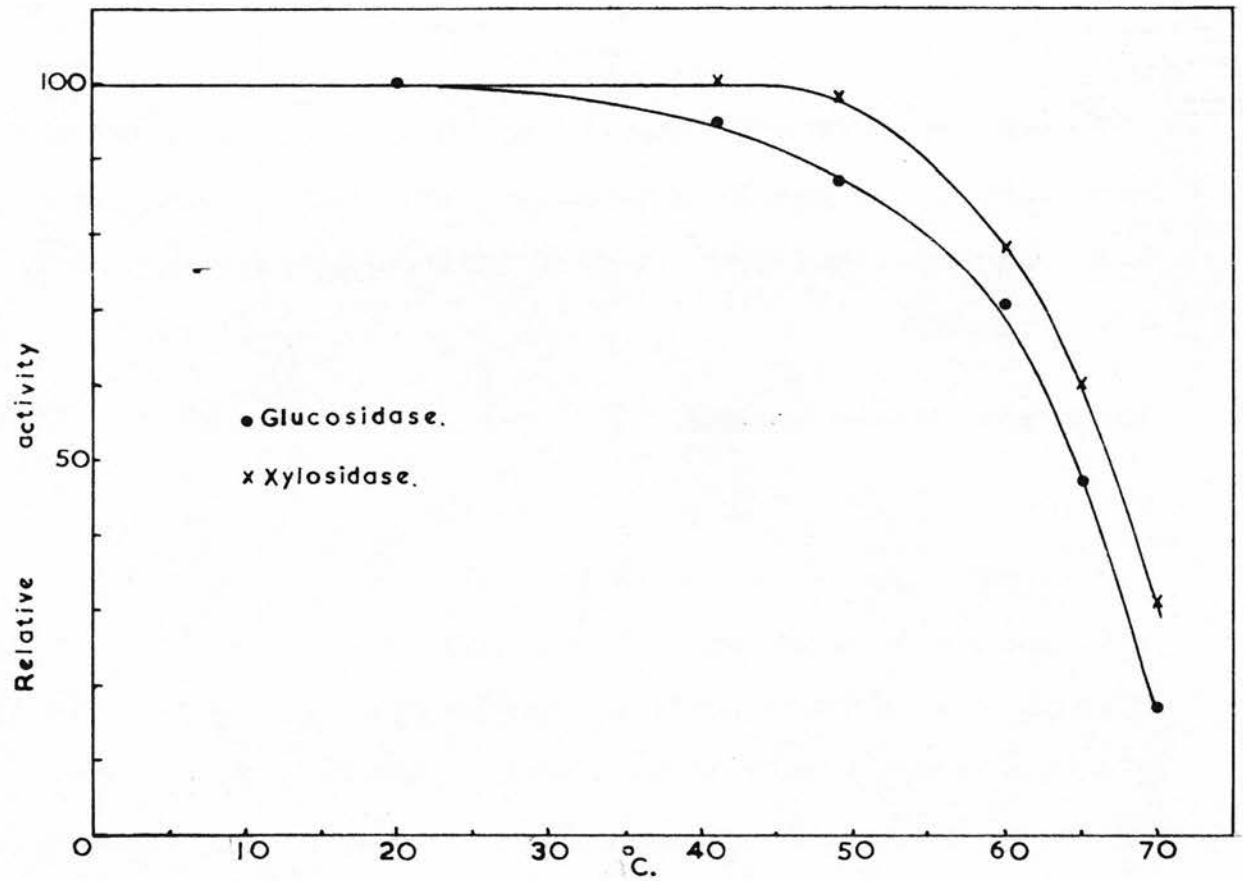


FIGURE 18

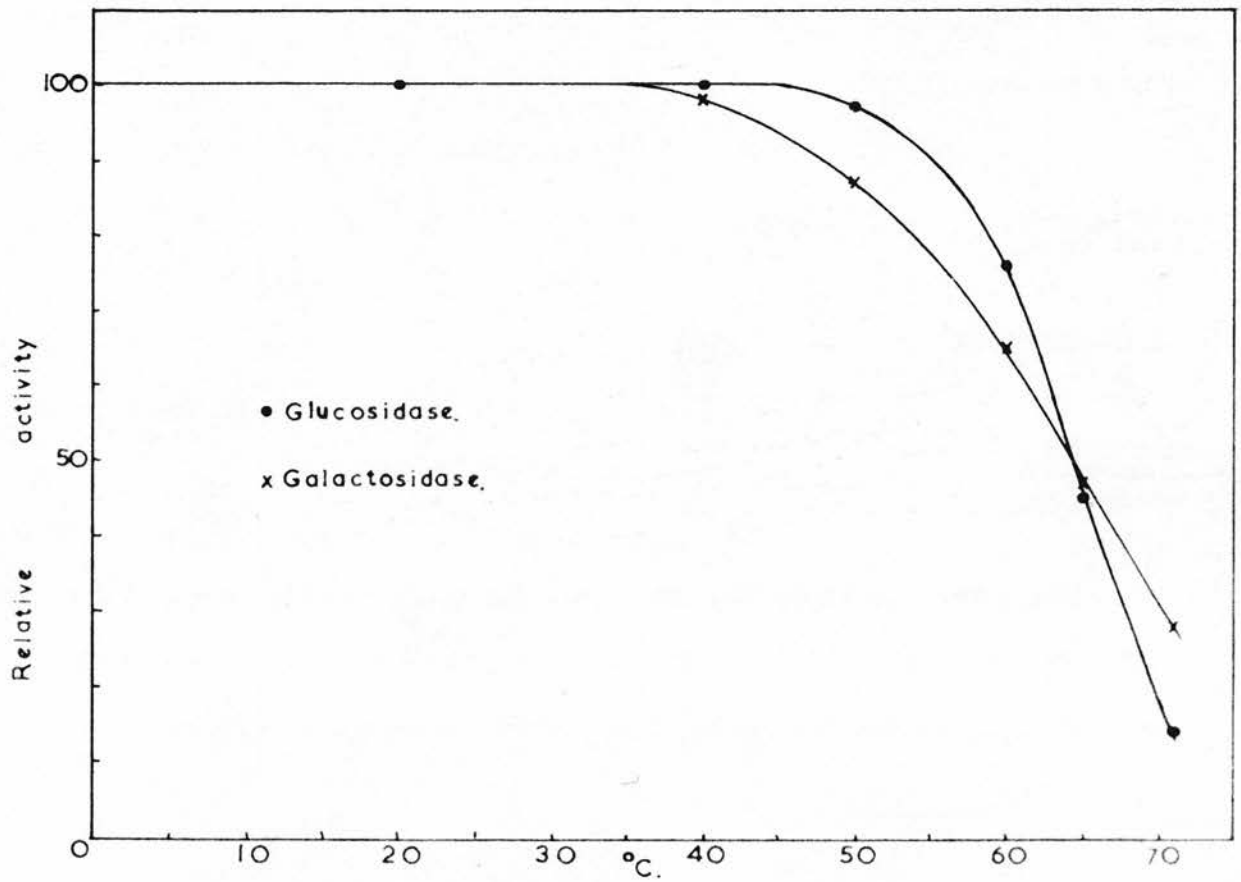


FIGURE 19

These results (Fig. 18) show the xylosidase to be substantially more stable to heat than the glucosidase. This adds further support to the suggestion that these two activities in Cladophora are due to separate enzymes.

Glucosidase and galactosidase.

An extract solution containing 30 mg./ml. was used. Samples (4 ml.) were heated at the required temperature for 15 min. 2 Ml. of this solution was used in the standard 5 ml. digest for the galactosidase assay and 2 ml. of a solution prepared by diluting 1 ml. of the stock solution with 2 ml. water used in a similar digest for the assay of glucosidase activity. The digests were incubated at 36°C. for 4 days. Extinction results are quoted below.

Temperature (°C.)	20	40	50	60	65	71
Glucosidase						
	0.735	0.737	0.717	0.557	0.328	0.100
Relative activity	100	100	97	76	45	14
Galactosidase						
	0.182	0.179	0.159	0.119	0.086	0.052
Relative activity	100	98	87	65	47	28

These results (Fig. 19) show that the galactosidase has a different heat deactivation pattern to that of the glucosidase, supporting the suggestion that the two activities are due to separate enzymes.

Comparing the two heat deactivation curves (Figs. 18 and 19) for the glucosidase activity it is apparent that this activity is more heat stable in more concentrated solutions.

Rhodymenia

Digests similar to those previously described were used, in which the extract solutions contained 10 mg./ml. Samples (2 ml.) of the extract solution were heated for 15 min. at the required temperature, cooled to room temperature, and the substrate solution and appropriate buffer added. Incubation was at 36°C. for 1 day in the glucosidase and xylosidase assays and 2 days in the galactosidase assay. In view of the pH/activity results for the galactosidase activity obtained above similar digests were incubated at pH 4.0 and 6.0. All other digests were at pH 6.0. Extinction results are quoted below.

Temperature (°C.)	20	41	50	60	65	70
Glucosidase						
	0.327	0.166	0.095	0.01	0.00	0.00
Relative activity	100	51	29	2	0	0
Xylosidase						
	0.130	0.050	0.014	0.00	0.00	0.00
Relative activity	100	39	11	0	0	0
Temperature (°C.)	20	39	49	59	64	74
Galactosidase						
pH 4.0						
	0.910	0.913	0.900	0.785	0.508	0.162
Relative activity	100	100	99	86	56	18
pH 6.0						
	0.720	0.705	0.676	0.558	0.348	0.162
Relative activity	100	98	94	78	48	23

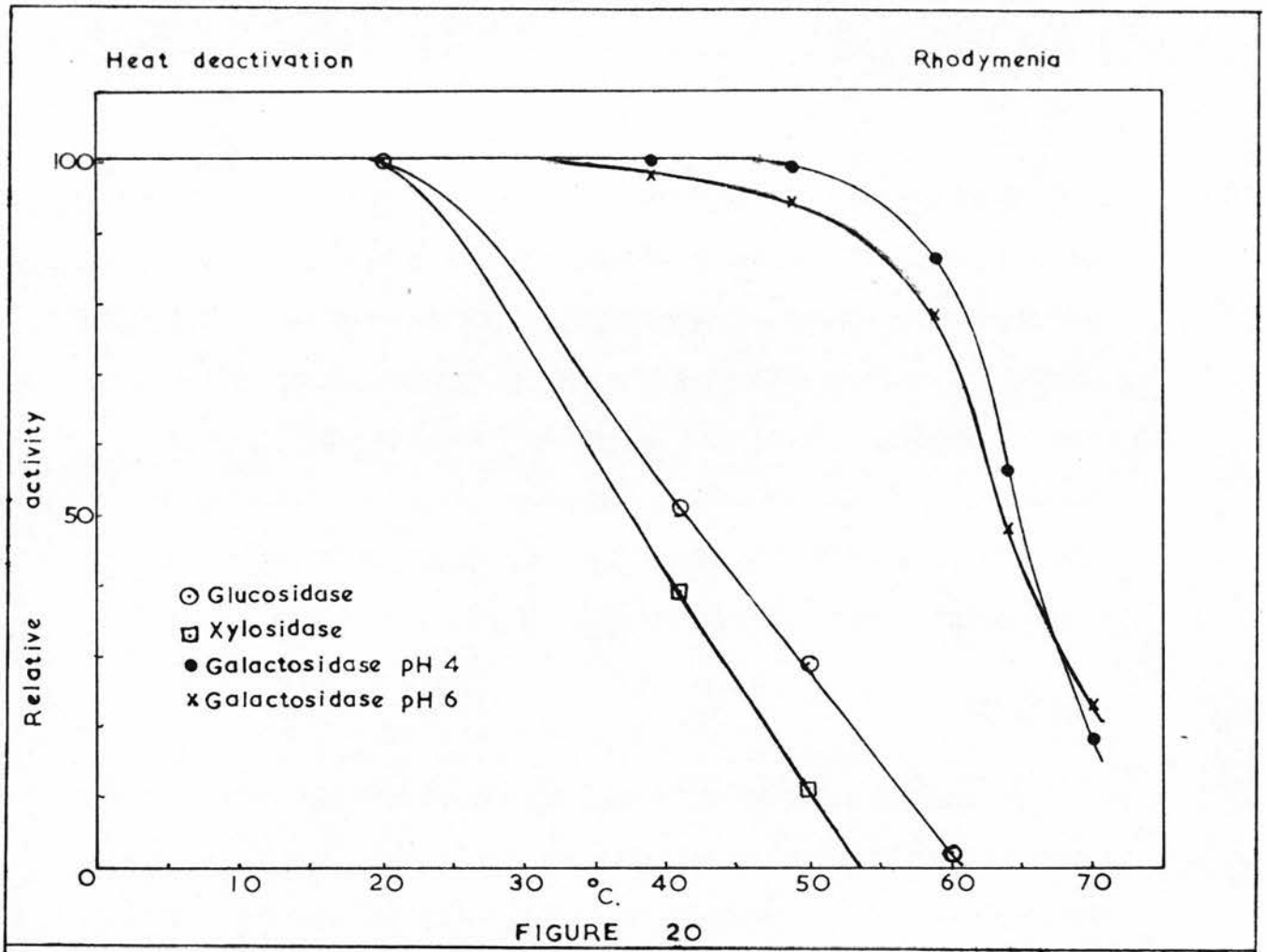


FIGURE 20

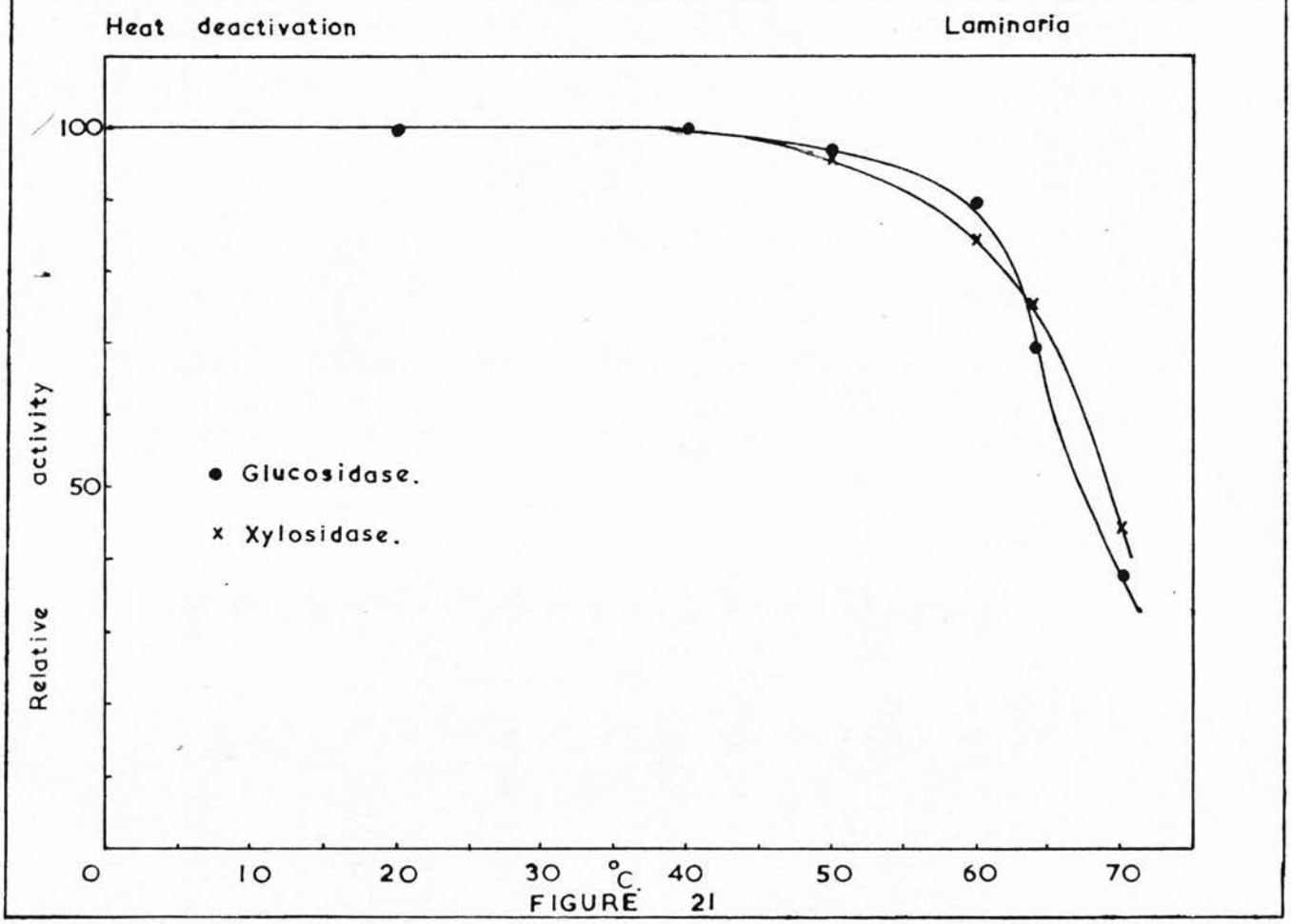


FIGURE 21

Consideration of these results (Fig. 20) confirms the suggestion made on the grounds of differing pH/activity curves that there is more than one glycosidase enzyme in Rhodymenia. As with the pH/activity curves there is a marked difference between the galactosidase and the glucosidase and xylosidase. There is a slight difference in the rates of deactivation of the xylosidase and glucosidase activities which considered alone is of doubtful significance. Similarly it is difficult to distinguish between the two possible galactosidase activities.

Laminaria

An extract solution of 10 mg./ml. was used. Samples (5 ml.) were heated at the appropriate temperature for 15 min., cooled to room temperature and 2 ml. samples examined in digests containing 2 ml. substrate and 1 ml. buffer pH 5.2. After 4 days incubation at 36°C. the liberated p-nitro-phenol was estimated in the usual way. Extinction results are quoted below.

Temperature (°C.)	20	40	50	60	64	70
Glucosidase						
	0.281	0.282	0.274	0.253	0.196	0.107
Relative activity	100	100	97	90	70	38
Xylosidase						
	0.168	0.167	0.161	0.142	0.127	0.075
Relative activity	100	100	96	85	76	45

A plot of these results (Fig. 21) shows that it is impossible to distinguish between the heat deactivations of the glucosidase and xylosidase activities. Thus, contrary to those in Cladophora and Rhodymenia the glycosidase activities of Laminaria cannot be readily distinguished by pH/activity or heat deactivation measurements.

Examination of the inhibitory effect of aldonolactones on the algal glycosidases.

Inhibition by aldonolactones has been used by a number of workers (e.g. Conchie, 1954; Reese and Mandels, 1959) to compare the specificity of glycosidase activity from various sources.

Glucono 1-5 lactone and galactono 1-4 lactone were used as inhibitors. Lactone solutions (0.02 M) were prepared and kept for four days to reach an approximate equilibrium. The glyconic acid in the equilibrium mixture was neutralised with dilute sodium hydroxide to the pH of the projected digest and samples added to these digests to examine the inhibitory effect of the residual lactone. Again the inhibitory effect of the lactone solution on the emulsin preparation was investigated for comparison. In all cases the final lactone concentration in the assay digest was less than 3 mM.

Rhodymenia

Comparison of the effect of glucono 1-5 lactone on the β-glucosidase and β-xylosidase activities.

Digests containing extract solution (10 mg./ml.), substrate solution (2 ml.), B.D.H. Universal buffer pH 6.0 (1 ml.) and lactone solution or water (1 ml.) were prepared and incubated at 36°C. The activities were assayed in the usual way, extinction results at 400 mμ being quoted below.

Incubation time (hr.)	22		46	
Digest	β-glucosidase	β-xylosidase	β-glucosidase	β-xylosidase
lactone present	0.196	0.111	0.281	0.166
lactone absent	0.344	0.124	0.501	0.179
% inhibition	43	10	44	7

These results confirm that the β-glucosidase and β-xylosidase activities of Rhodymenia are due to separate enzymes. Had they been due to the same or very similar enzymes the percentage inhibition should have been the same in both digests. (cf. emulsin results below).

Comparison of the effect of glucono 1-5 lactone and galactono 1-4 lactone on the β-glucosidase and β-galactosidase activities.

Digests similar to those in the previous experiment were prepared with buffer at pH 5.5. After incubation at 37°C. for 42 hr. the extinction results shown were obtained.

Digest	Extinction	% inhibition
Galactoside/water	0.472	-
Galactoside/galactonolactone	0.118	75
Galactoside/gluconolactone	0.475	0
Glucoside/water	0.338	-
Glucoside/gluconolactone	0.207	40
Glucoside/galactonolactone	0.308	10

The glucosidase activity is strongly inhibited by the added gluconolactone, while this lactone has no effect on the galactosidase activity. Similarly the galactosidase activity is inhibited by the galactonolactone which has only a weak effect on the glucosidase activity. This adds further confirmation to the suggestion of distinct glycosidase activities in Rhodymenia.

Cladophora

Digests similar to those above were prepared with extract solution (20 mg./ml.) and buffer pH 6.0. Glucono 1-5 lactone was used as the inhibitor. After incubation at 36°C. for 44 hr. the extinction results quoted below were obtained.

Digest	β-glucosidase	β-xylosidase
lactone present	0.442	0.194
lactone absent	0.580	0.236
% inhibition	24	18

These results are not so conclusive as those for Rhodymenia above but in conjunction with the pH/activity and heat deactivation results previously obtained they support the presence of distinct glycosidase activities in Cladophora.

Laminaria

Digests similar to those above were prepared with extract solution (20 mg./ml.) and buffer pH 5.5. After incubation for 6 days at 36°C. the following extinction results were obtained.

Digest	β-glucosidase	β-xylosidase
lactone present	0.045	0.040
lactone absent	0.246	0.117
% inhibition	82	66

Thus some indication of separate glycosidase activity in the Laminaria extract has been obtained by this method although these activities were inseparable by pH/activity and heat deactivation experiments.

Emulsin

Digests similar to those above were prepared at pH 6.0. A solution of emulsin (2 mg./ml.) was used for the xylosidase digest and a thirty-fold dilution used for the glucosidase digest. Glucono 1-5 lactone was used as the inhibitor. Digests were incubated at 35°C. for 30 min. and the extinction results quoted below obtained.

Digest	β-glucosidase	β-xylosidase
lactone present	0.170	0.141
lactone absent	0.286	0.239
% inhibition	40	41

Rhodymenia lactase. pH/activity curve.

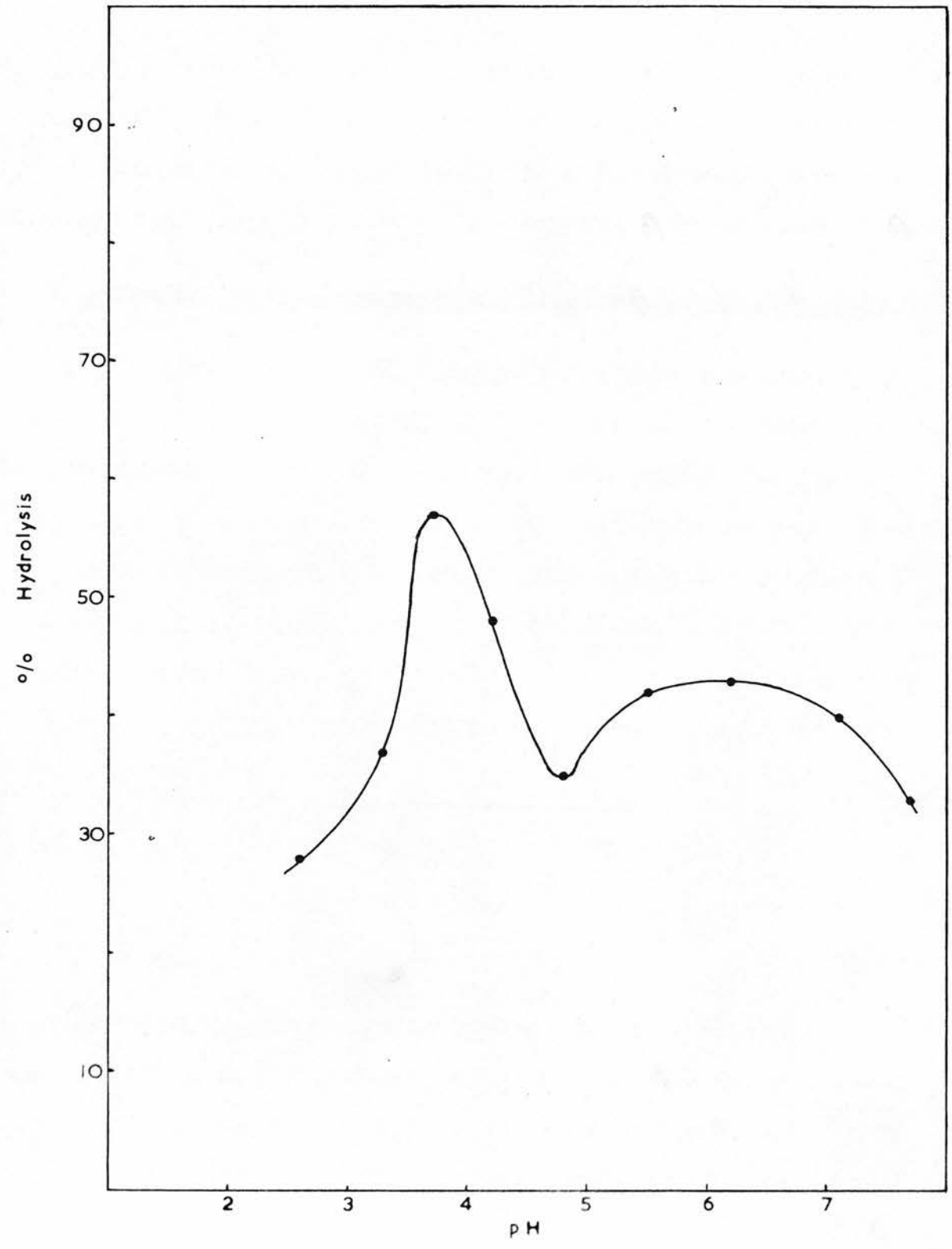


FIGURE 22

These results confirm that the glycosidase activities in emulsin are due to the same or very similar enzymes. Using glucono and galactono 1-4 lactones Heyworth and Walker (1962) obtained similar results for the β-glucosidase and β-galactosidase activities of sweet almond emulsin.

Investigation of the β, 1-4 galactosidase activity of Rhodymenia.

Digests were prepared containing 2 ml. extract solution (10 mg./ml.), 2 ml. lactose solution (0.0005 M), 1 ml. buffer pH 2.6 - 7.7 and 1 ml. water and incubated at 37°C. for 3 days. The reducing sugar content of each digest was determined by the method of Park and Johnson described in Methods and Materials. The results below are quoted as extinction values at 700 mμ. The values for a lactose control and a lactose hydrolysate were also obtained. This latter control represents 100% hydrolysis of the available lactose in the assay digests.

pH	2.6	3.3	3.8	4.2	4.8	5.5	6.2	7.1	7.7
	0.398	0.424	0.479	0.455	0.417	0.437	0.442	0.432	0.412

Lactose control, 0.320; hydrolysate control, 0.600.

% hydrolysis	28	37	57	48	35	42	43	40	33

Comparing these results plotted in Fig. 22 with those from digests with p-nitrophenyl-β-D-galactoside as substrate (Fig. 16) the presence of the second β-galactosidase activity previously suggest is apparent. This has optimum activity at about pH 6 and will be referred to as the β, 1-4 galactosidase. The other, with optimum activity at about pH 4 will be referred to as the aryl β-galactosidase.

Investigation of a possible inhibitory effect of glucose and xylose on the β-glucosidase and β-xylosidase activities of Rhodymenia.

Solutions (0.004 M, 1 ml./digest) of glucose and xylose were added to digests containing 2 ml. extract solution (10 mg./ml.), 2 ml. substrate solution and 1 ml. B.D.H. Universal buffer pH 6.0. Control digests containing water instead of glucose or xylose were also prepared. After 21 hr. incubation at 36°C. the following extinction results were obtained.

Digest			
Glucoside/water	0.398	Xyloside/water	0.196
Glucoside/glucose	0.386	Xyloside/glucose	0.203
Glucoside/xylose	0.394	Xyloside/xylose	0.201

The added glucose or xylose have no significant effect on the glycosidase activities, at concentrations twice that of the substrate.

Discussion

The pH optima obtained for the algal glycosidases, with the exception of the Rhodymenia aryl β-galactosidase, range from pH 5.2 - 6.3, and compare with values reported for glycosidase activity from other sources. Jermyn (1959) in an investigation of the β-glucosidase activity of fungi obtained a range of pH optima from 4.3 - 5.9 with an average value of 4.7 or 4.8. Dahlqvist (1961) reported an optimum pH of 6.0 for the β-glucosidase and β-galactosidase of pig intestinal mucosa, and Heyworth and Walker (1962) reported 5.5 and 5.6 as the

respective optima for the same two activities from sweet almond emulsin. These latter figures agree with that of 5.5 obtained in the present work for the β-glucosidase and β-xylosidase activities from the same source.

The Rhodymenia aryl β-galactosidase has the more unusual pH optimum of 4, but this is not without precedent. Hill (1934) quoted a pH optimum of 3.4 for the β-galactosidase from Lucerne seeds. Hutson and Manners (1963) have obtained values between pH 4.5 and 5.0 for optimum β-glycosidase activities from the same source. Helferich and Vorsatz (1935) reported an optimum range of 3.5 - 4.0 for the β-galactosidase of coffee bean emulsin.

The glycosidase activities examined in the algal extracts are, in the main, readily distinguishable as separate enzymes. The greatest differentiation is between the aryl β-galactosidase and the β-glucosidase and β-xylosidase of Rhodymenia, the pH optima and the heat deactivation patterns being completely different. The β-glucosidase and β-xylosidase from this extract have virtually identical pH/activity curves but the slight variation in the heat deactivation patterns and the more marked differences in the inhibitory effect of glucono 1-5 lactone on the two activities show that these activities are due to separate enzymes. Further investigation of the β, 1-4 galactosidase was impracticable in the crude extract since the aryl β-galactosidase showed appreciable hydrolytic activity towards lactose.

The glycosidase activities in Cladophora have distinct pH/activity curves and this, together with the variation of activity ratios in different extract fractions, small variations in the rates of heat

deactivation and the different inhibitory effect of glucono 1-5 lactone confirm that the glycosidases of this alga too are due to separate enzymes.

The Laminaria glycosidases are not so readily distinguished. The β-galactosidase activity was so weak that it could not be readily examined. The β-glucosidase and β-xylosidase have identical pH/activity curves with the exception of the secondary optimum at pH 8. The weakness of this activity relative to the generally weak activity of the extract made the further investigation of this secondary activity impracticable. Heat deactivation patterns obtained for the β-glucosidase and β-xylosidase were not significantly different but the variation in the inhibitory effect of glucono 1-5 lactone suggests that the glycosidases of Laminaria are, like the other algae examined, due to separate enzymes.

These findings for the algal glycosidases contrast with those for sweet almond emulsin by Heyworth and Walker (1962) and in this chapter which show that the glycosidase activities of this source cannot be distinguished by pH/activity, heat deactivation or aldono-lactone inhibition experiments. Similar inability to achieve separation of glycosidase activities by these means was reported by Dahlqvist (1961) and Heyworth and Dahlqvist (1962) in investigations of the β-glucosidase and β-galactosidase of pig intestinal mucosa.

The failure of a two-fold excess of glucose and xylose to inhibit the β-glucosidase and β-xylosidase activities of Rhodymenia extracts puts these activities in the "emulsin" category of glycosidase activities proposed by Ezaki (1940) and Horikoshi (1942), the other division being

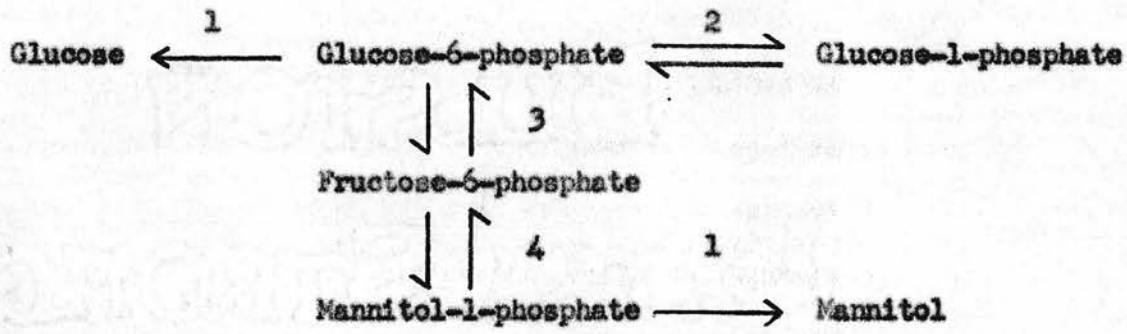
the "taka-type" where the glycosidase activity is inhibited by the appropriate aldose. It is now generally believed that these categories represent extremes of a range, rather than an absolute difference (Jermyn 1961), but the results obtained show some similarity between the algal extracts and the almond emulsin used for comparison.

The results obtained in this chapter have shown that, in contrast to the glycosidase activities from other sources, the algal glycosidases are, in general, readily distinguished as separate enzymes.

Chapter IV

HEXOSE METABOLISM IN CLADOPHORA RUPESTRIS, RHODYMENIA PALMATA, LAMINARIA CLOUSTONI AND FUCUS VESICULOSUS.

The work described in this chapter constitutes a survey and preliminary examination of the enzymes, present in algal extracts, taking part in the scheme outlined below:-



The hydrolysis of phosphate esters (1) may be catalysed by specific phosphatases or by non-specific phosphomonoesterases. Truly specific glucose-6-phosphatase occurs only in mammalian tissue (Swanson, 1955) while a specific mannitol-1-phosphatase has been reported in the fungus Piricularia oryzae by Yamada et al (1961). Non-specific phosphomonoesterases occur in great variety in plants and animals. They may be classified by their optimum pH range and by the effect of magnesium ions on the activity. By this means Roche (1950) has divided these enzymes into four 'types'.

I Optimum pH 8.6 - 9.4; activated by magnesium ions.

II Optimum pH 5.0 - 5.5; no effect by magnesium ions.

III Optimum pH 3.4 - 4.2; inhibited by magnesium ions.

IV Optimum pH 5.0 - 6.0; activated by magnesium ions.

In general he states that alkaline phosphomonoesterase activity is confined to animal tissue and acid activity to plant tissue. More recent reports support this generalisation. Naganna et al (1955) were unable to demonstrate alkaline phosphomonoesterase activity in potato extracts and Pathak and Sreenivasan (1955) obtained similar results from the moulds Aspergillus niger and A. oryzae. Acid phosphomonoesterases have been demonstrated in wheat leaf and mushroom extracts (Roberts, 1963; Dehennin et al 1961).

Algal phosphatase activity has not been extensively examined. Sosa-Bourdouil (1946) reported phosphatase activity in the antherozoids and ovules of Fucus vesiculosus but did not specify the pH of the assay. Desruisseaux and Baudoin (1949) investigated the phosphomonoesterase activity of the calcareous red alga Corallina officinalis demonstrating two major activities with optimum activities at pH 5.6 - 6.2 and at pH 9.0 and a weak activity at pH 3.6 - 4.6. Brandes and Elston (1956) demonstrated alkaline phosphatase activity during histochemical studies on Chlorella vulgaris. Duncan (1956) reported alkaline phosphatase activity in extracts of Cladophora rupestris.

The enzyme catalysing the equilibrium reaction 2, phosphoglucomutase, occurs in mammalian tissue and in yeast (Cori et al 1938a). It has been reported in potato extracts (Boser, 1957) Jack beans (Cardini, 1951) and in Phaseolus radiatus, a species of bean, by Ramasarma et al (1954). Strong phosphoglucomutase activity has also been reported in the phloem sap of Robinia pseudoacacia by Wanner (1954). Jacobi (1957a) was unable to detect phosphoglucomutase in the green alga Ulva lactuca.

Phosphoglucose isomerase, the enzyme catalysing the equilibrium reaction 3 has been demonstrated in a number of sources. The enzyme extracted from muscle has optimum activity at pH 9.0 (Slein, 1955) whereas the yeast enzyme has a pH optimum of 7.8 - 8.0 (Noltmann and Bruns, 1959). Singh (1959) reported a similar optimum pH range for the enzyme from the mould Aspergillus niger while Ramasarma and Giri (1956) reported a variation in pH optima with the substrate, pH 7.8 with glucose-6-phosphate and pH 9.0 with fructose-6-phosphate, using extracts of Phaseolus radiatus.

The conversion of fructose-6-phosphate to mannitol-1-phosphate (4) has been demonstrated in the fungus Piricularia oryzae by Yamada et al (1961). The reaction was dependent on diphosphopyridine nucleotide (DPN or DPNH) and was shown to be reversible in the presence of the appropriate co-factor. Similar DPN-dependent mannitol-1-phosphate reductase activity has been demonstrated in bacteria by Kaplan and his co-workers (Wolff and Kaplan, 1956; Liss et al 1962). In view of the established presence of mannitol as a major component of the low molecular weight carbohydrate of brown algae (Lindberg, 1956) it is possible that this pathway may be demonstrable in extracts of brown algae, free mannitol being liberated by the action of a mannitol-1-phosphatase on mannitol-1-phosphate derived from fructose-6-phosphate.

Experimental

Standard digests containing extract solution, substrate solution, buffer (1 ml. of each) and water or ionic solution (2 ml.) were incubated at 37°C. for appropriate periods. Any variation in this composition is noted in the text.

The algal extracts used were: Cladophora, extract C and a homogenate prepared as required from fresh seaweed stored at -15°C.; Rhodymenia, extracts P and C; Laminaria; Fucus vesiculosus, a homogenate prepared as required from fresh seaweed stored at -15°C.

Seaweed homogenates were prepared by homogenizing the wet seaweed (10 g.) in distilled water (100 ml. at 0°C.) in an ice-bath for 2-3 min. During homogenizing the temperature of the extract rose to about 10°C. The pH was 6.5 - 7.0. The Cladophora extract was centrifuged at 2420 g. and the Fucus extract at 25,000 g. for 10-15 min. before use. This latter high speed centrifugation was necessary to remove the mucilaginous suspension of alginate and other material in the homogenate.

The pH range 2.6 - 10.5 was covered by 0.1 M citric acid/sodium citrate (2.6 - 4.8), 0.2 M tris-maleate (5.5 - 7.7), and 0.2 M glycine (8.6 - 10.5) buffers.

Phosphomonoesterase activity

Commercially available sodium β-glycerophosphate was used in substrate solutions (1.25 mg./ml.). In 2 ml. digest samples removed for inorganic phosphorus estimation (Methods and Materials) a net extinction of 1.00 at 725 mμ represents 100% hydrolysis.

Activities in the algal extracts were surveyed over the pH range 2.6 - 10.5. In view of the marked relationship between magnesium ions and phosphatase activity from other sources (Roche, 1950) the effect of these ions on the algal phosphatase activities was also examined.

To check the validity of phosphorus estimation as a measure of enzymic activity standard digests were prepared with Cladophora extract C (various concentrations) at pH 9.2, containing magnesium ions to a final concentration of 10 mM. After incubation for 24 hr. inorganic phosphorus was estimated. Extinction values at 725 mμ are given below.

Extract solution (mg./ml.)	2.5	5.0	7.5	10.0	15.0	20.0
Extinction	0.094	0.144	0.211	0.250	0.354	0.416

A plot of these results shows a linear relationship between extinction and extract concentration in the range 2.5 - 15.0 mg./ml. extract solution confirming that, within this range, inorganic phosphorus estimation gives a valid measure of enzymic activity.

Cladophora

Digests were prepared with a homogenate and incubated for 20 hr. Control digests containing extract or substrate solution alone were also prepared. After incubation, 2 ml. samples were withdrawn for the estimation of inorganic phosphorus according to the procedure in Methods and Materials. The results are quoted below as extinction values at 725 mμ. (E).

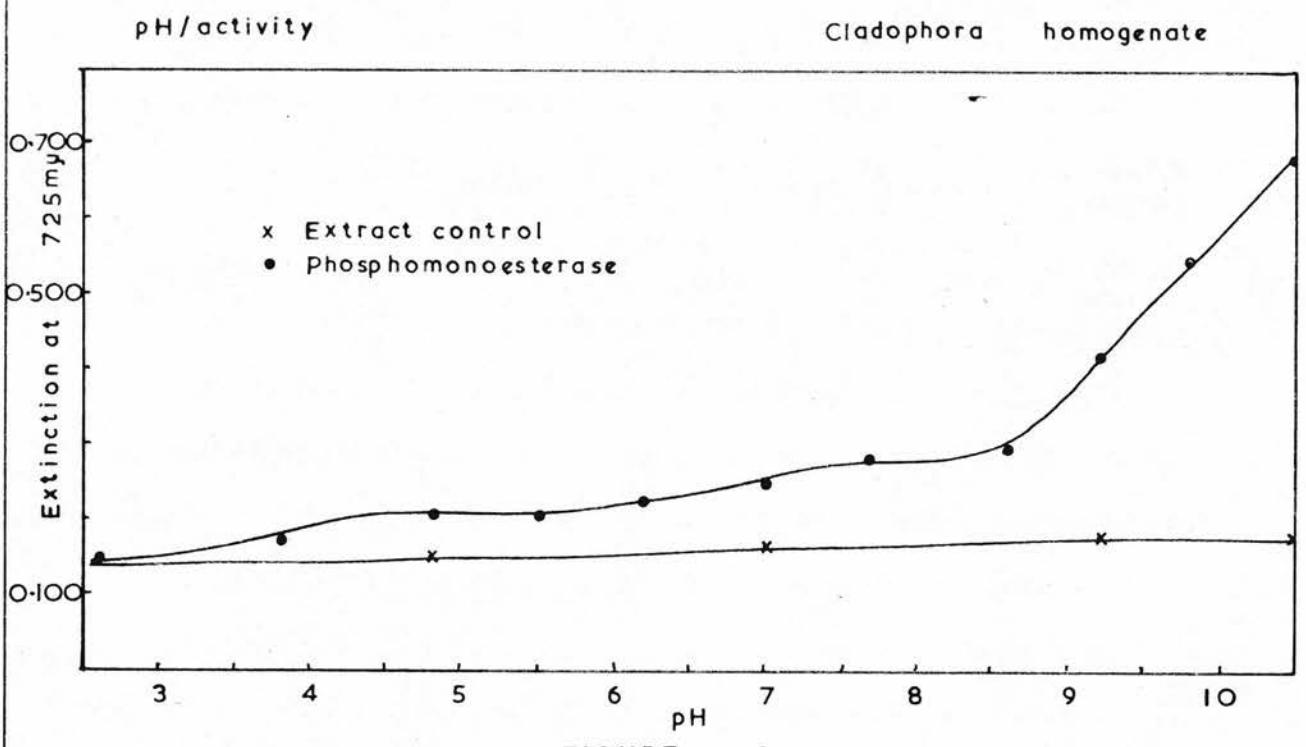


FIGURE 23

Alkaline phosphomonoesterase. Heat control.

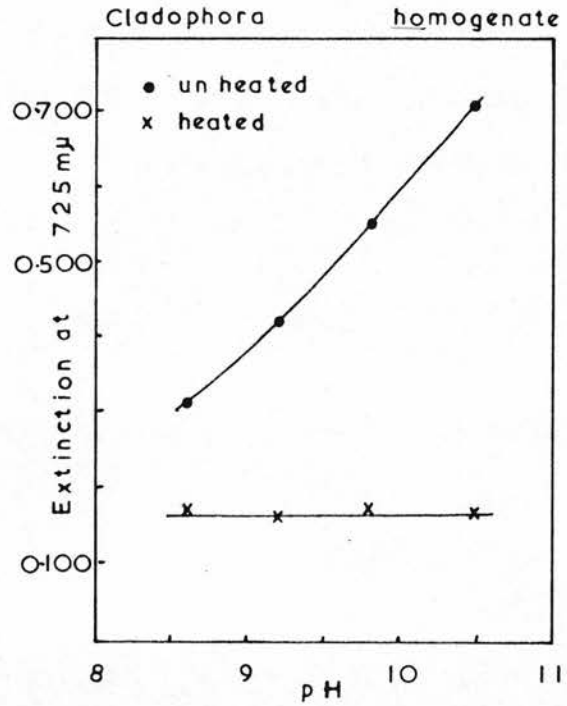


FIGURE 24

pH	2.6	3.8	4.8	5.5	6.2	7.0	7.7	8.6	9.2	9.8	10.5
E	0.149	0.170	0.206	0.206	0.223	0.249	0.283	0.292	0.417	0.542	0.678
Extract control			0.149			0.166			0.177		0.176
Substrate control		0.009		0.013					0.008		0.015

The slight variation in the extract control is unavoidable in using a direct homogenate as enzyme source as some phosphate esters may be expected in the crude extract. The absence of any significant change in the substrate controls indicates that the substrate is stable in the buffered solutions.

In view of the unusual activity of this preparation in alkaline solution a control experiment was carried out, examining the activity in the pH range 8.6 - 10.5 using a deactivated homogenate (15 min. at 98°C.) to confirm that the activity was due to enzymic hydrolysis. Standard digests were prepared and after 24 hr. incubation the activity was determined in the usual way. Extract and substrate controls were run at pH 10.5. Extinction results are quoted below.

pH	8.6	9.2	9.8	10.5
E	0.314	0.422	0.552	0.709
Extract control				0.171
Substrate control				0.008
E, heated control	0.170	0.157	0.161	0.156
Extract control				0.153
Substrate control				0.008

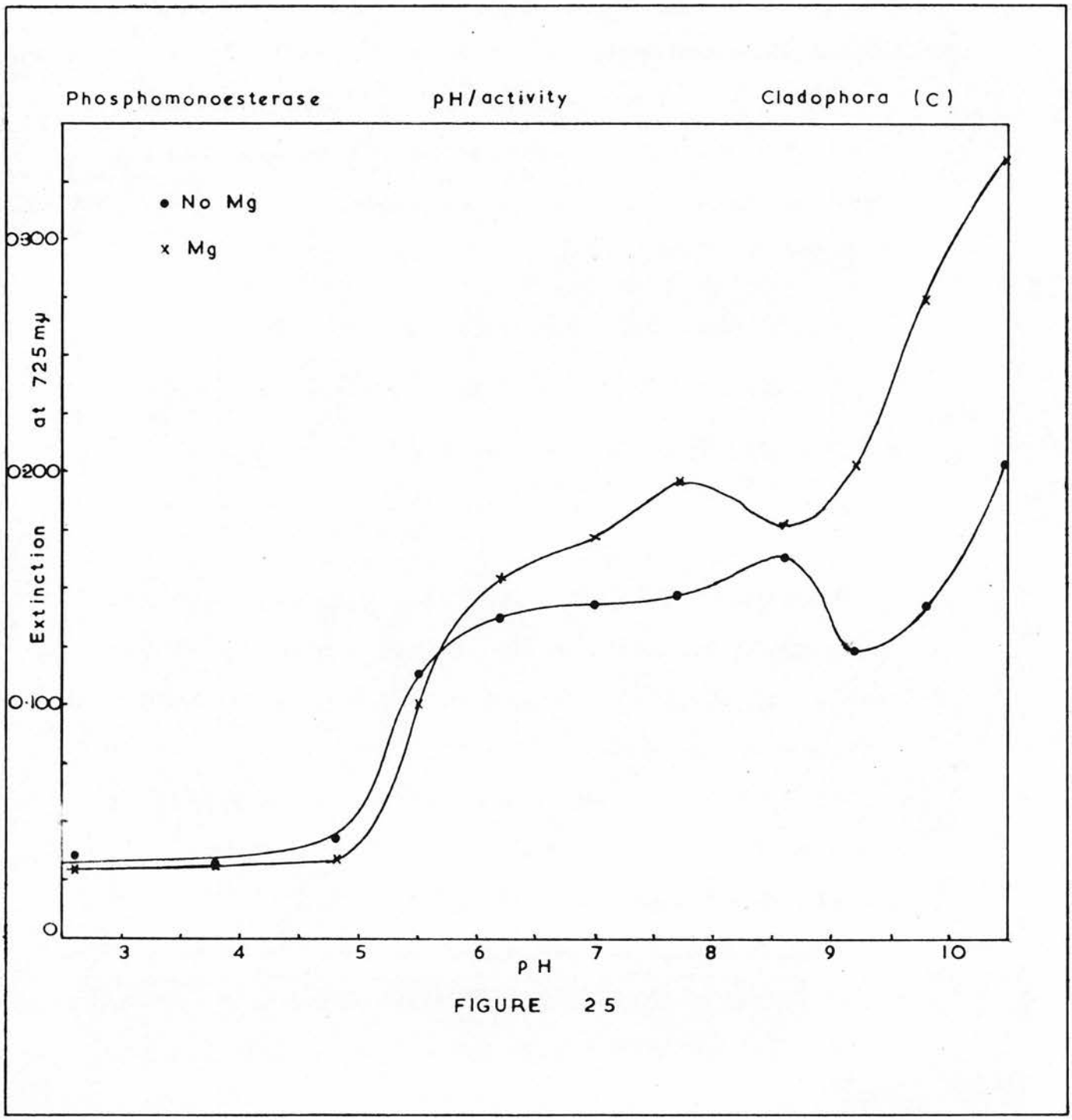

Phosphomonoesterase
pH/activity
Cladophora (C)
• No Mg
x Mg
Extinction at 725 mμ
0·300
0·200
0·100
0
3
4
5
6
7
8
9
10
pH

FIGURE 25

These results indicate that this alkaline phosphatase activity is, in fact, due to enzymic hydrolysis. The results of these experiments with the Cladophora homogenate are shown diagramatically in Figs. 23 and 24.

The activity of a solution of extract C (10 mg./ml.) was investigated in the presence and absence of added magnesium ions (magnesium chloride, final concentration 10 mM.). The results obtained are quoted as extinction values, after 20 hr. incubation.

pH	2.6	3.8	4.8	5.5	6.2	7.0	7.7	8.6	9.2	9.8	10.5
without Mg	0.036	0.032	0.043	0.113	0.137	0.143	0.147	0.163	0.123	0.142	0.202
with Mg	0.029	0.030	0.034	0.100	0.154	0.172	0.196	0.180	0.202	0.273	0.332
Extract control						0.030 0.025					

These results are shown in Fig. 25. Comparing the results from the Cladophora homogenate and from extract C (Figs. 23 and 25) it is apparent that the main feature, especially in the fresh extract, is the strong activity at unusually alkaline pH, Roche (1950) quoting an optimum pH range of 8.6 - 9.4 for normal alkaline phosphatase from animal and plant sources which, like the algal phosphatase, is strongly activated by magnesium ions. At the acid end of the pH range the fresh extract appears to contain weak acid phosphatase activity and in view of the slight deactivation effect due to magnesium observed at pH 4.8 and 5.5 in the extract C experiment it is probable that this activity is due to a normal phosphomonoesterase type III (Roche, 1950) found in plants and animals, with optimum pH range 3.4 - 4.2 and inhibited by magnesium ions. In view of the weak activity and contamination

with other stronger activities, this activity in the fresh extract was not further investigated. The phosphatase activity with optimum pH ranging from 6.2 - 7.7, relatively stronger in extract C than in the fresh extract will be shown later to be due to a probable glucose-6-phosphatase activity. The apparent optimum at pH 8.6 in the extract C experiment in the absence of magnesium ions is probably caused by the summation of two separate activities and is masked by the magnesium activation effect in the other experiment. This summation effect makes the interpretation of results from examination of crude extracts difficult, in that small peaks are produced in the pH/activity curve which might be wrongly attributed to weak, but separate activities.

The effect of magnesium ion concentration on the phosphatase activity of extract C was investigated at pH 9.8. After incubation for 8 hr. the following extinction results were obtained.

Final Mg ion concentration (mM)	0	5	10	15	20	25	30	40
E	0.102	0.162	0.177	0.180	0.195	0.198	0.198	0.210
Enzyme control	0.032							

This represents an immediate increase in activity of almost 100% on the addition of magnesium with a slower increase as the magnesium ion concentration is raised.

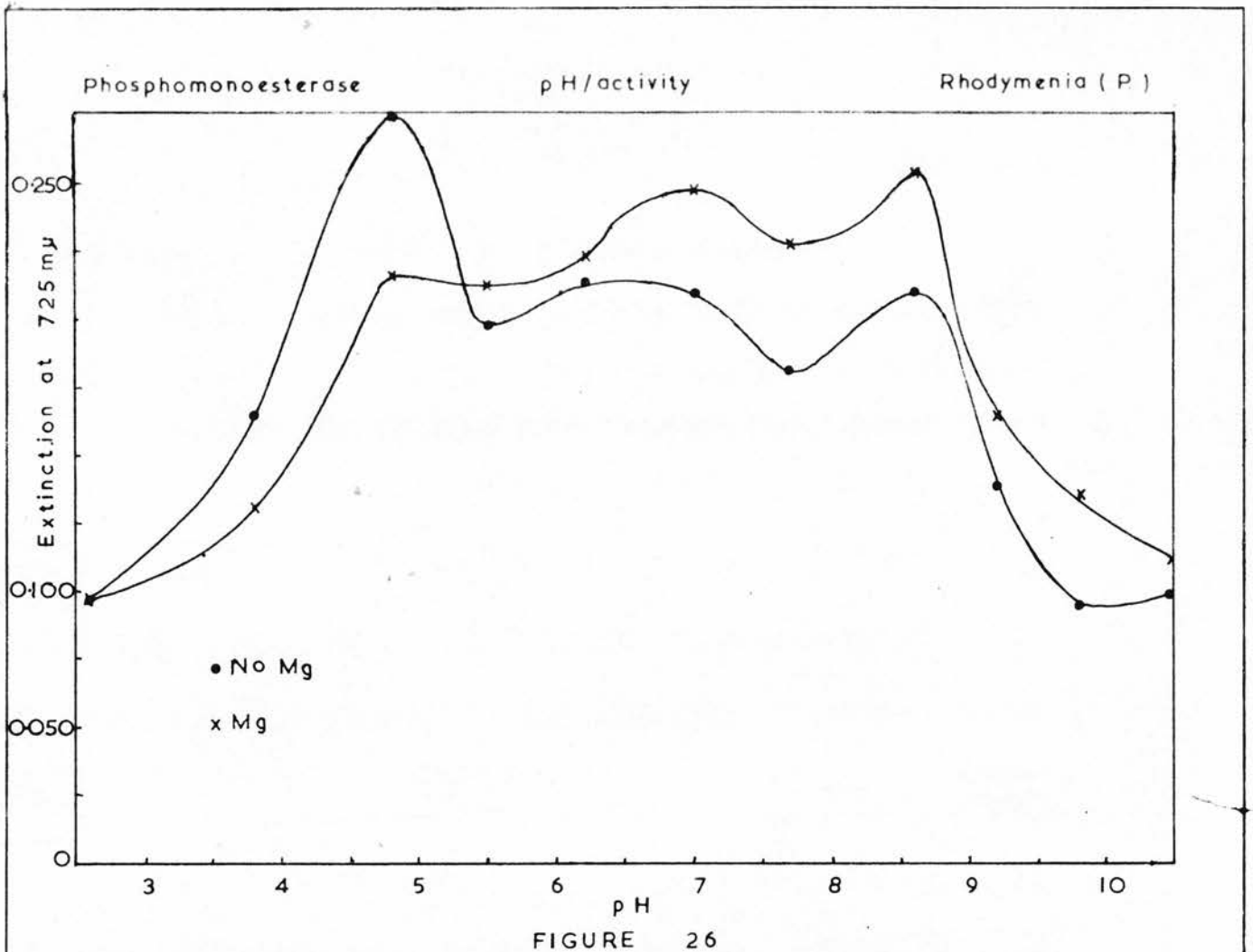


FIGURE 26

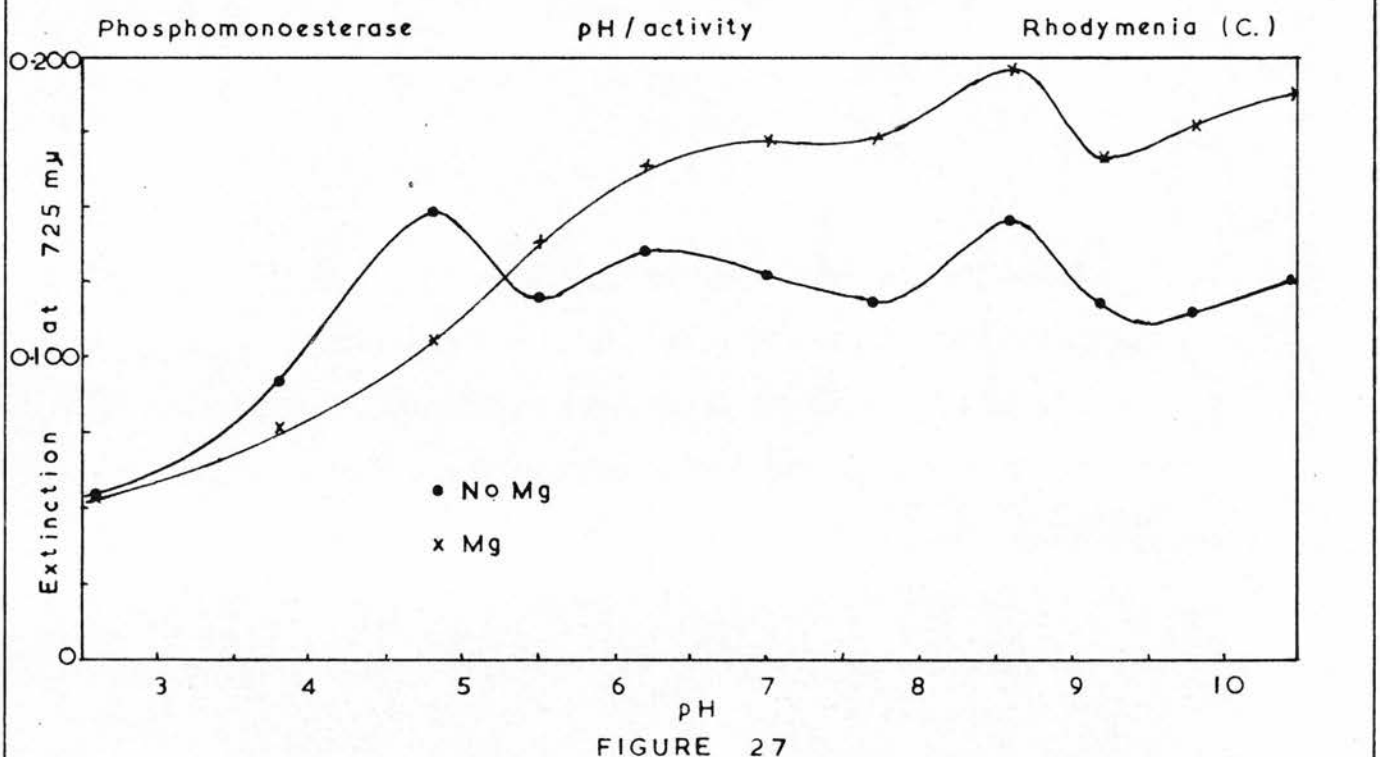


FIGURE 27

Rhodymenia

The activity of both extract P and C (10 mg./ml.) was investigated in the presence and absence of added magnesium ions as in the experiments with Cladophora extract C. Both extracts were incubated for 42 hr. and the extinction results reported below obtained.

Extract P

pH	2.6	3.8	4.8	5.5	6.2	7.1	7.7	8.6	9.2	9.8	10.5
without Mg	0.097	0.165	0.276	0.197	0.213	0.208	0.181	0.210	0.138	0.094	0.098
with Mg	0.097	0.130	0.215	0.212	0.222	0.246	0.226	0.252	0.163	0.134	0.110
Extract control						0.083 0.084					

Extract C

pH	2.6	3.8	4.8	5.5	6.2	7.0	7.7	8.6	9.2	9.8	10.5
without Mg	0.055	0.092	0.148	0.120	0.135	0.127	0.118	0.145	0.118	0.115	0.125
with Mg	0.053	0.077	0.106	0.138	0.164	0.172	0.173	0.196	0.167	0.177	0.188
Extract control						0.052 0.058					

These results from extracts P and C are shown in Figs. 26 and 27. Comparison of these two Figures indicates the presence in Rhodymenia extracts of at least three phosphatase activities. The first of these, present in both extracts, has pH optimum of 4.8 and is inhibited by magnesium ions. This activity falls within the phosphomonoesterase type III classification mentioned above. The other activity present in both extracts has pH optimum of 8.6 and is activated by magnesium ions,

making this activity similar in properties to those of the phosphomonoesterase type I with optimum pH ranging from 8.6 - 9.4 and activated by magnesium ions. The third activity, present only in extract C is similar to the alkaline phosphatase found in the Cladophora extracts above. This activity has an optimum pH of 10.5 or higher and is activated by magnesium. The identification of a fourth phosphatase activity with optimum pH at about pH 7 is doubtful in view of the summation effect mentioned previously and the presence of strong activity on either side of this peak.

Laminaria

Extract solutions (20 mg./ml. 2 ml.) were used in otherwise standard 5 ml. digests in experiments similar to those described for the two algae above. After incubation for 43 hr. the following extinction results were obtained.

pH	2.6	3.8	4.8	5.5	6.2	7.1	7.7	8.6	9.2	9.8	10.5
without Mg	0.007	0.012	0.008	0.020	0.038	0.067	0.060	0.065	0.074	0.047	0.032
with Mg	0.016	0.006	0.010	0.018	0.024	0.057	0.042	0.047	0.044	0.032	0.018
Extract control						0.006 0.008					

These results are shown in Fig. 28. There are two weak activities apparent, one with pH optimum at about pH 7 and the other at about pH 9. As these activities are shown independently by the two experiments they may be taken as significant, but in view of the very weak activity the apparent general inhibitory effect of magnesium ions must be regarded with caution.

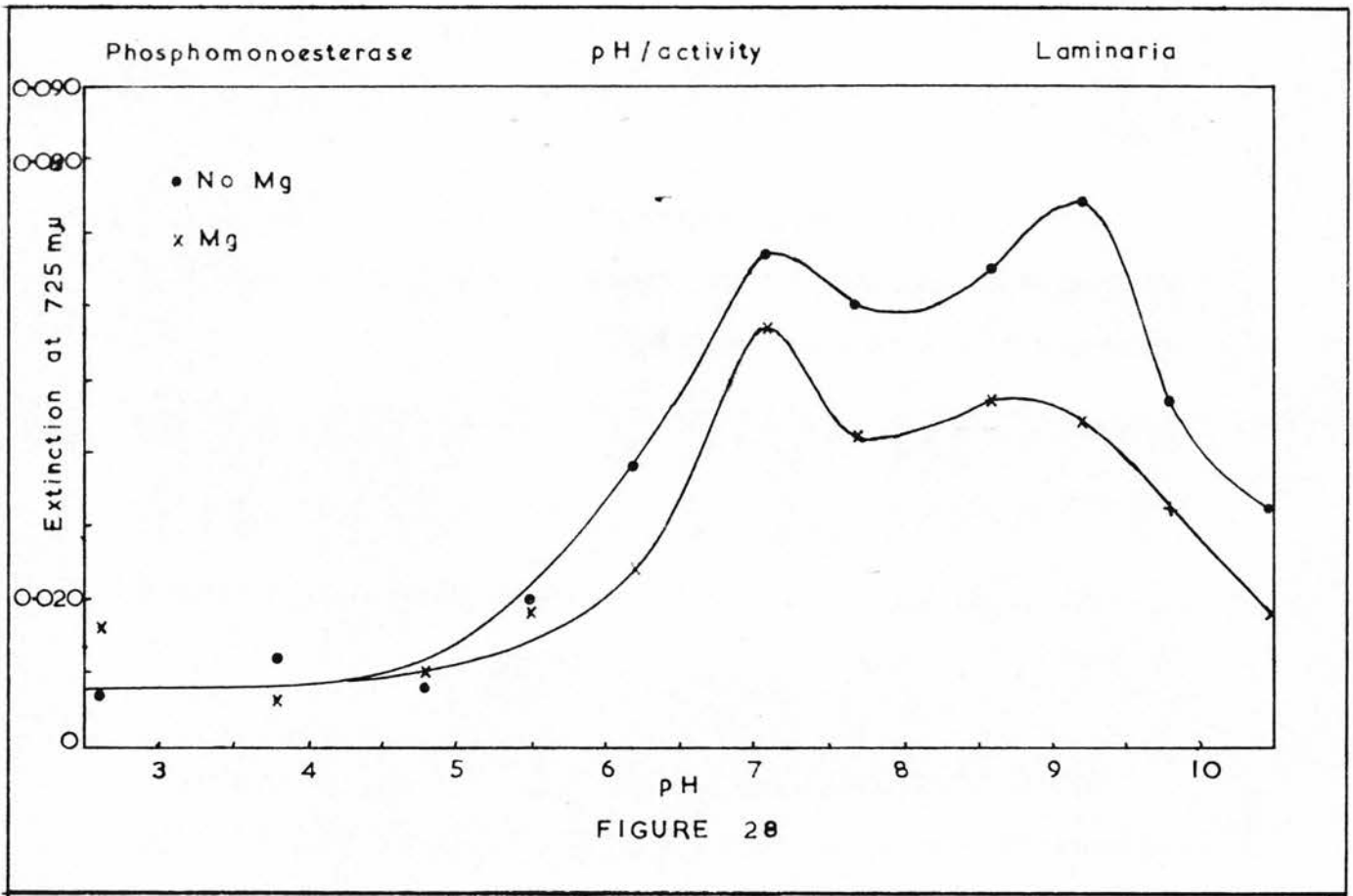


FIGURE 28

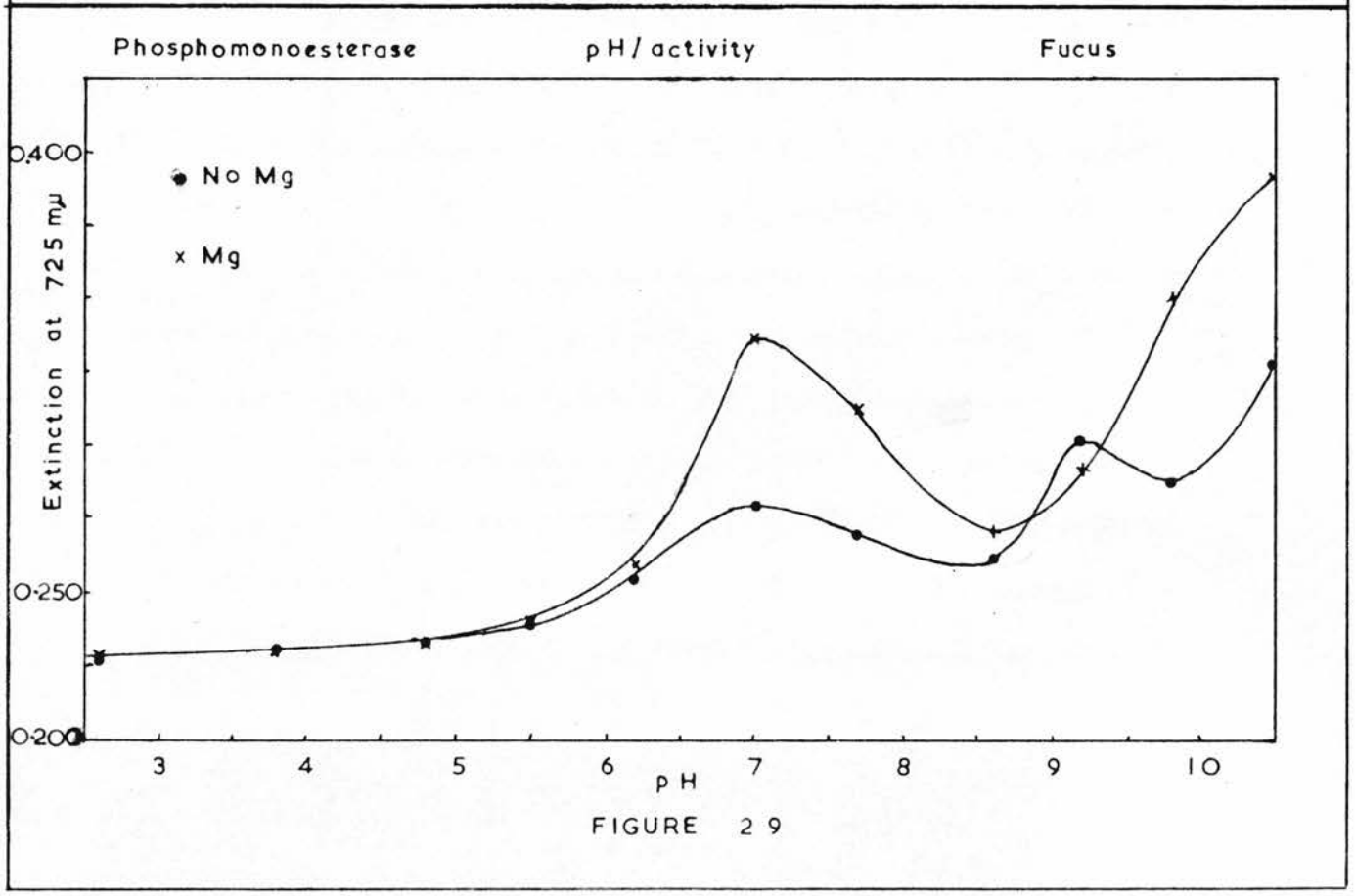


FIGURE 29

Fucus

Using a freshly prepared homogenate, experiments similar to those above were carried out. After incubation for 42 hr. the extinction results quoted below were obtained.

pH	2.6	3.8	4.8	5.5	6.2	7.0	7.7	8.6	9.2	9.8	10.5
without Mg	0.227	0.232	0.234	0.239	0.255	0.280	0.270	0.262	0.302	0.288	0.328
with Mg	0.230	0.230	0.232	0.243	0.259	0.337	0.313	0.271	0.292	0.351	0.392
Extract control						0.240 0.232					

These results are shown in Fig. 29. In carrying out these experiments it was noted that the digests assumed a brownish colouration varying in intensity with pH from almost colourless at pH 4.8 to a rich brown at pH 10.5. That this colour does not affect the inorganic phosphorus estimations is shown by a complete range of extract controls run with the experiments investigating the mannitol-1-phosphatase activity of the Fucus extract described below.

Considering the results in Fig. 29 two phosphatase activities are evident, both of which are activated by magnesium ions. One has optimum activity at about pH 7 and the other is similar to the alkaline phosphatase demonstrated above in Cladophora and Rhodymenia extracts, with optimum pH of 10.5 or greater. Again there is evidence of a weak activity of doubtful significance with optimum activity at pH 9.2.

Glucose-6-phosphatase survey.

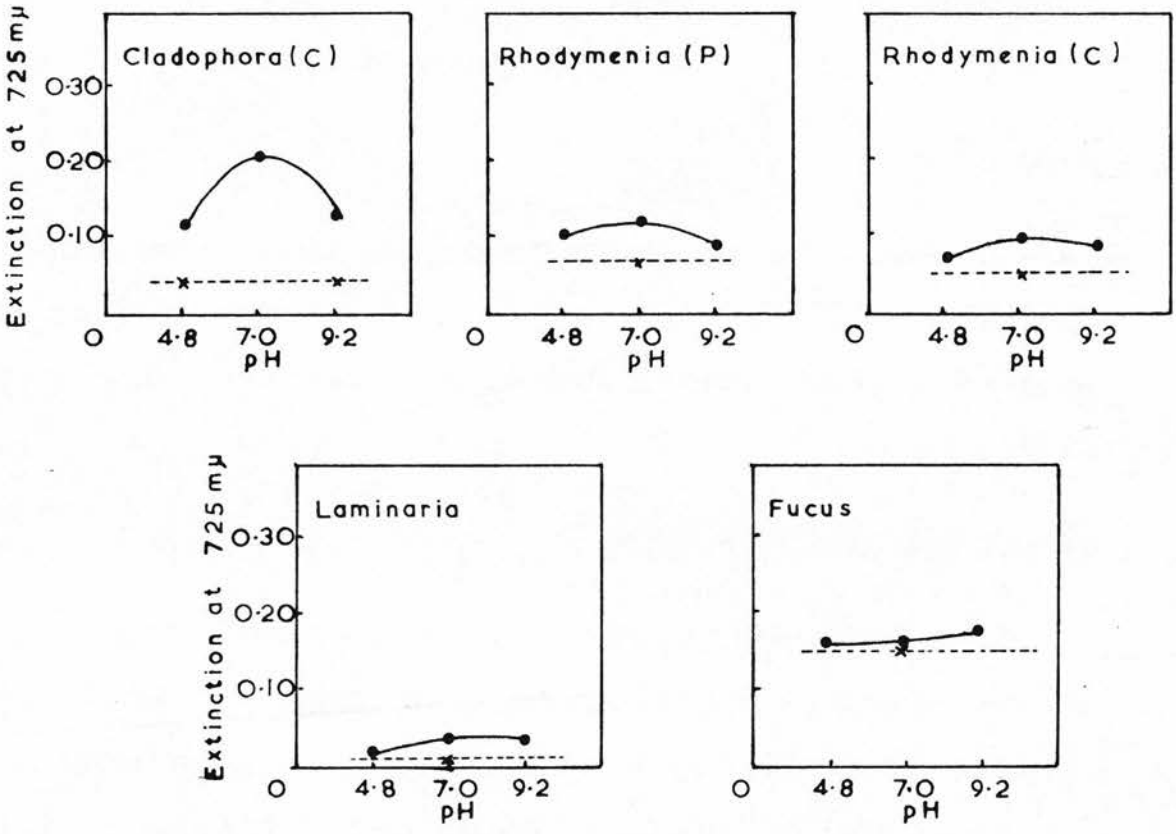


FIGURE 30

For reference purposes the phosphomonoesterase activity of almond emulsin (Methods and Materials) was investigated in digests at pH 4.8, 7.0 and 9.2. Emulsin solutions containing 1 mg./ml. were used in standard digests incubated for 46 hr. The extinction results obtained are quoted below.

pH	4.8	7.0	9.2
without Mg	0.620	0.330	0.018
with Mg	0.572	0.318	0.016
Extract		0.014	
control		0.014	

Thus the almond emulsin contains a strong phosphomonoesterase type III activity. This is in agreement with a report by Bredereck et al (1936) of a phosphatase from almond emulsin with optimum activity at pH 4.5 - 5.0.

Glucose-6-phosphatase activity

Using the standard digest the hydrolytic activity of the various extracts towards glucose-6-phosphate was investigated. The substrate solutions contained 1.5 mg./ml. glucose-6-phosphate di-potassium salt di-hydrate, which in 2 ml. digest samples taken for inorganic phosphorus estimation would give a net extinction at 725 mμ of 1.00 representing 100% hydrolysis. Algal extract solutions contained 10 mg./ml. with the exception of the Laminaria extract (20 mg./ml.) and the homogenates.

The results of a survey of the indicated extracts are shown in Fig. 30. Digests were incubated for 24 hr. at pH 4.8, 7.0 and 9.2 and 2 ml. samples examined for inorganic phosphorus in the usual way.

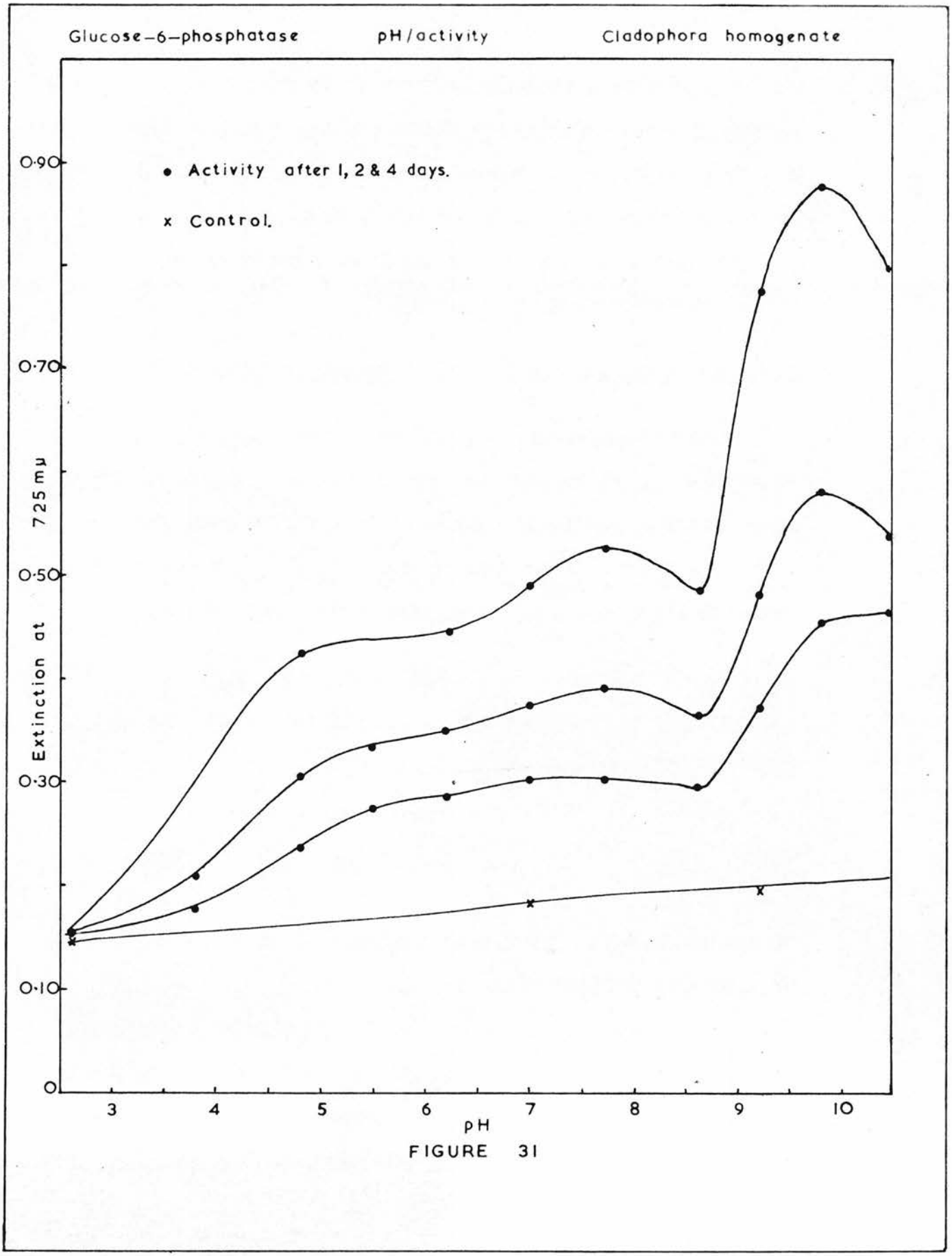

Glucose–6–phosphatase pH/activity Cladophora homogenate
• Activity after 1, 2 & 4 days.
x Control.
Extinction at 725 mμ
0·90
0·70
0·50
0·30
0·10
0
3
4
5
6
7
8
9
10
pH

FIGURE 31

The level of extract controls is shown by the dotted lines. Only the Cladophora extract C shows reasonable activity possibly differing from that obtained in the investigations above with sodium β-glycerophosphate. The further examination of this activity is described below. Investigation of the activity of the other extracts towards glucose-6-phosphate was not extended.

Glucose-6-phosphatase activity of Cladophora extracts

Standard digests were prepared containing Cladophora homogenate. After 1 and 2 days, samples were removed and the phosphatase activity assayed in the usual way. The 4 day extinction results also quoted below were obtained from a similar but separate experiment designed to examine the phosphoglucose isomerase activity described later.

pH	2.6	3.8	4.8	5.5	6.2	7.0	7.7	8.6	9.2	9.8	10.5
1 day	0.153	0.178	0.236	0.273	0.284	0.300	0.300	0.293	0.368	0.452	0.461
2 day	0.157	0.209	0.303	0.334	0.348	0.372	0.388	0.362	0.477	0.578	0.452
4 day	0.158	-	0.422	-	0.442	0.488	0.522	0.481	0.771	0.871	0.793
Extract controls	0.144					0.180			0.192		

The extract control figures were obtained from the 4 day experiment. These results are plotted in Fig. 31.

The effect of magnesium ions on this phosphatase activity was examined in standard digests with extract C solutions (10 mg./ml.). Magnesium chloride was added as in previous experiments to a final concentration of 10 mM. After 24 hr. incubation the following extinction results were obtained.

pH	2.6	3.8	4.8	5.5	6.2	7.0	7.7	8.6	9.2	9.8	10.5
without Mg	0.071	0.077	0.100	0.250	0.327	0.332	0.318	0.314	0.210	0.220	0.263
with Mg	0.070	0.077	0.083	0.218	0.301	0.326	0.324	0.317	0.278	0.349	0.340
Extract control						0.069 0.068					

These results are shown in Fig. 32.

Comparing the results with glucose-6-phosphate as substrate (Figs. 31 and 32) with those with sodium β-glycerophosphate as substrate (Figs. 23 and 25) it is apparent that an enzyme hydrolysing glucose-6-phosphate preferentially is present in the Cladophora extracts. This is demonstrated, especially in the extract C digests, by the marked variation in the relative activities of the alkaline phosphatase and the neutral phosphatase when the substrate is altered.

Magnesium ions have no significant effect on the extract C glucose-6-phosphatase activity. The differing activities of the two Cladophora extracts in the pH range 2.6 - 4.8 again provides evidence for the existance of a separate acid phosphatase in the homogenate. Some further light is thrown on the alkaline phosphatase activity by the changing ratio of activity at pH 9.8 to that at pH 10.5 on prolonged incubation (Fig. 31). This suggests that although greater initial activity is evident at pH 10.5 the enzyme is unstable at this pH and deactivation during prolonged incubation reveals a true optimum activity at pH 9.8.

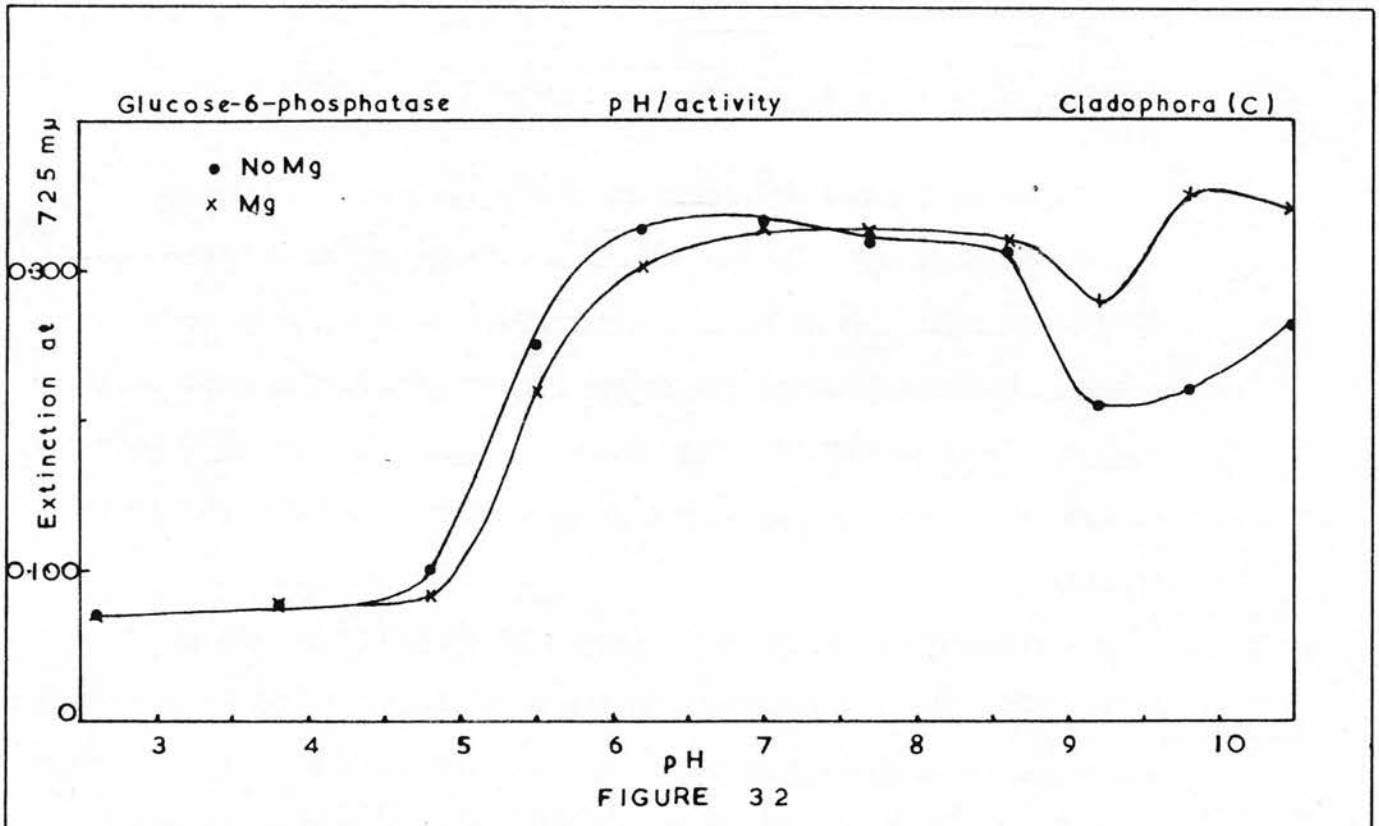


FIGURE 32

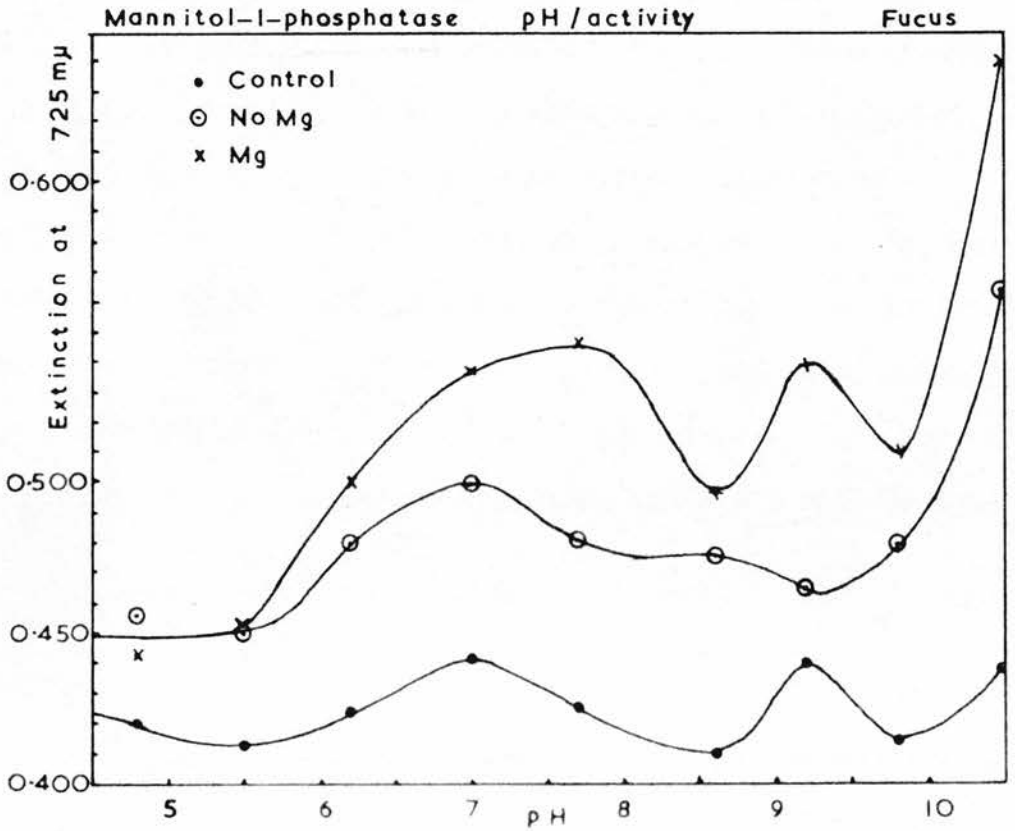


FIGURE 33

Mannitol-1-phosphatase activity

It is well known that mannitol occurs as a main constituent of the extractable low molecular weight carbohydrates of the brown algae (Lindberg, 1956) and as Yamada et al (1961) had reported a specific mannitol-1-phosphatase in the fungus Piricularia oryzae which contains mannitol as a constituent sugar the activity of the available extracts of the brown algae Laminaria and Fucus towards mannitol-1-phosphate was examined.

Mannitol-1-phosphate was prepared as described in Methods and Materials. The concentration of the stock solution was determined by the mannitol estimation method also described in Methods and Materials to be 2.59 mg./ml. expressed as the free mannitol-1-phosphoric acid. In phosphatase digests 1 ml. of a three fold dilution of this stock solution was used as the substrate solution. Thus a net extinction of 0.800 represents 100% hydrolysis.

To check that this preparation could be enzymically hydrolysed a standard digest containing an almond emulsin solution (1 mg./ml.) was incubated at pH 4.8 for 42 hr. Extract and substrate controls were prepared also, the latter being to obtain a correction for possible inorganic phosphate in the unpurified mannitol-1-phosphate preparation. The following extinction results were obtained:-

Digest 0.365; Extract control 0.013; Substrate control 0.030.

The mannitol-1-phosphate preparation was substantially hydrolysed (40%) by the acid phosphatase previously shown to be present in the emulsin preparation.

Fucus

Standard digests were prepared and incubated for 42 hr. Three series of digests were examined, one containing extract and substrate, another similar series containing magnesium chloride to a final concentration of 10 mM and a third extract control series. The extinction results obtained are quoted below.

pH	4.8	5.5	6.2	7.0	7.7	8.6	9.2	9.8	10.5
without Mg	0.457	0.450	0.480	0.499	0.481	0.476	0.465	0.479	0.564
with Mg	0.443	0.453	0.500	0.537	0.546	0.496	0.539	0.510	0.638
Extract control	0.420	0.412	0.424	0.442	0.425	0.410	0.440	0.415	0.438

Mannitol-1-phosphate controls also obtained were 0.030, 0.027 and 0.028.

These results are plotted in Fig. 33. The strong brown colour which develops in the more alkaline digests with Fucus extracts previously mentioned in connection with the experiments with these extracts and β-glycerophosphate, has no effect on the level of the enzyme control, which only reflects the variations in the effect of pH on the phosphatase activity of digests containing added substrate, utilising what phosphate esters are present in the homogenate. Comparing the results in Fig. 33 with those in Fig. 29 it is evident that no highly specific mannitol-1-phosphatase is present in the Fucus extract. The activity towards this substrate confirms the results from the previous experiments that this Fucus extract contains at least two phosphatase activities, one with optimum activity at about pH 7 and the other alkaline phosphatase

similar to that observed in the Cladophora and Rhodymenia extracts, both activated by magnesium ions.

Laminaria

The activity of the Laminaria extract (2 ml., 20 mg./ml.) towards the mannitol-1-phosphate was examined in otherwise standard 5 ml. digests, with and without added magnesium ions, incubated for 48 hr. Extinction results obtained are quoted below.

pH	4.8	5.5	6.2	7.0	7.7	8.6	9.2	9.8
without Mg	0.032	0.041	0.055	0.066	0.067	0.056	0.052	0.044
with Mg	0.027	0.042	0.043	0.054	0.053	0.052	0.056	0.047
Extract control				0.007 0.008				

Mannitol-1-phosphate controls gave 0.026 and 0.027.

The activity of this Laminaria extract is so low that little significance can be attached to these results. They show that the Laminaria extract does not contain a mannitol-1-phosphatase activity greater than that toward β-glycerophosphate previously examined. The apparent inhibitory effect of magnesium ions observed in the experiments with β-glycerophosphate is, in general, operative in these digests also.

As a result of these experiments with the brown algae preparations no evidence has been obtained for the existance of a specific mannitol-1-phosphatase.

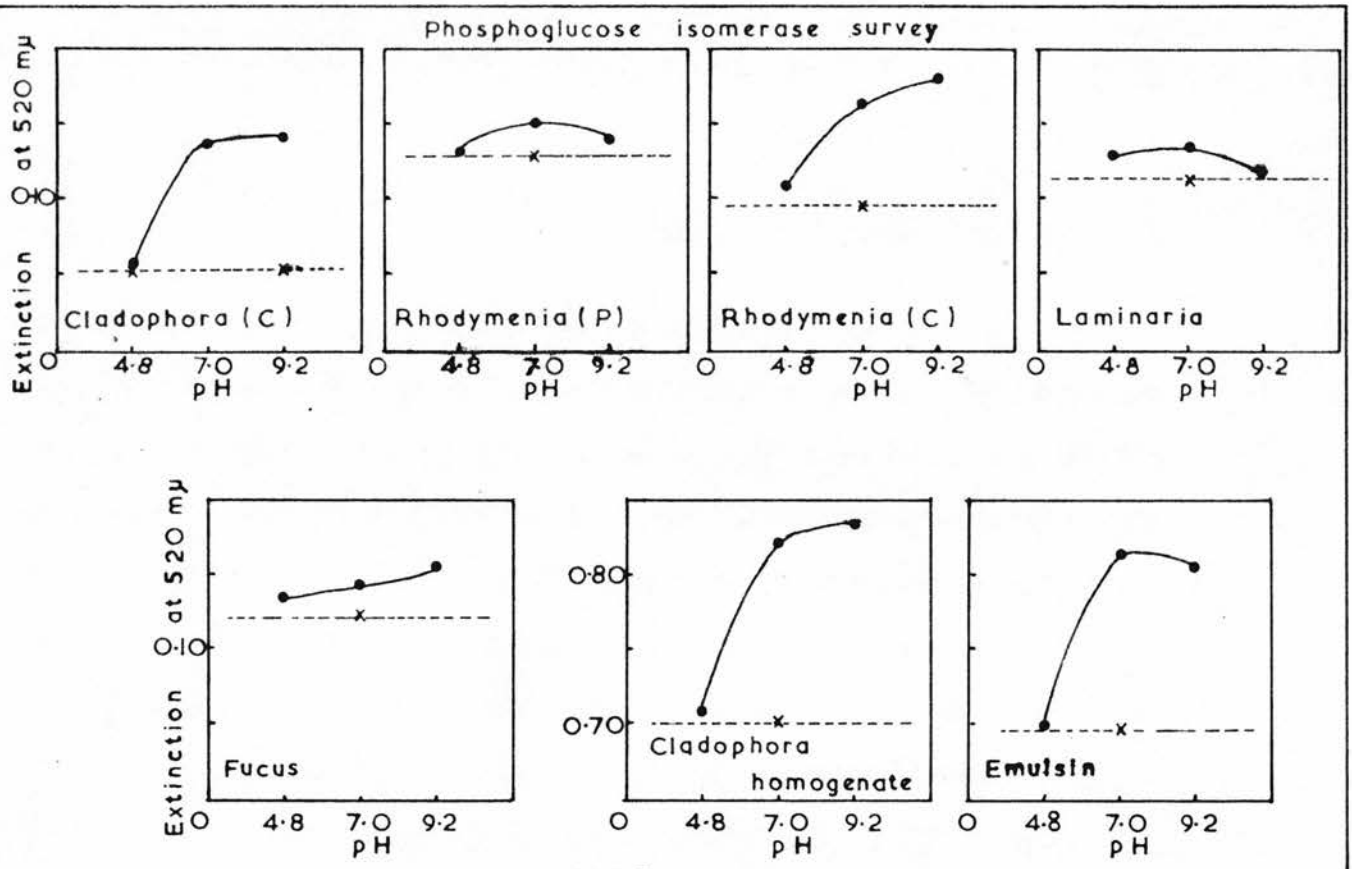


FIGURE 34

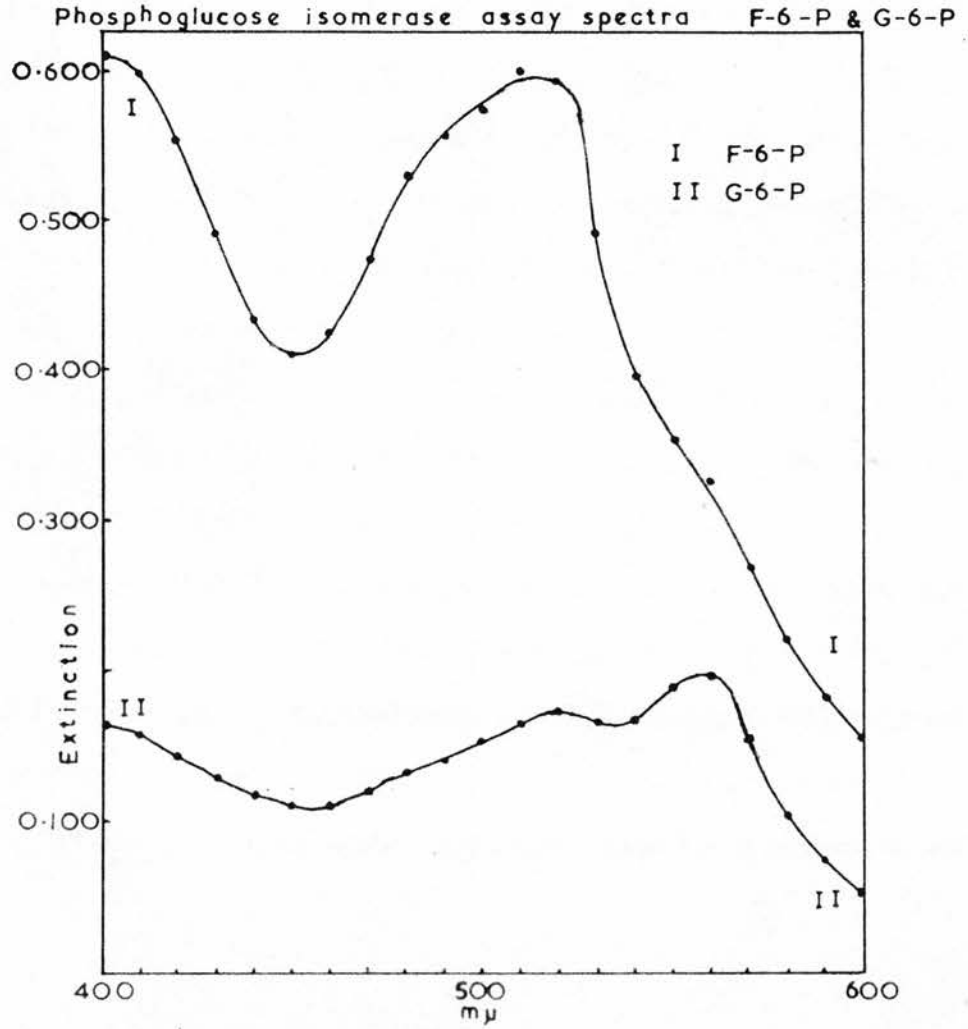


FIGURE 35

Phosphoglucose isomerase activity

The various algal extracts were examined for this activity in the same digests used for the glucose-6-phosphatase survey above. In addition, digests were prepared with Cladophora homogenate and with the almond emulsin preparation (extract solution, 1 mg./ml.). This latter digest was prepared in an attempt to find a plant source of high activity. After 26-28 hr. incubation the results shown in Fig. 34 were obtained as extinction values at 520 mμ. using the acid resorcinol fructose-6-phosphate assay method (Methods and Materials).

Only the Cladophora preparations, Rhodymenia extract C and the emulsin showed obvious activity but in carrying out this survey it was noted that the extract control assays developed a purple colour, especially in the Laminaria and Rhodymenia digests, which it was thought might interfere with the fructose-6-phosphate assay at 520 mμ. To check this the algal extracts were re-examined and the absorption spectra of the assay solutions between 400 and 600 mμ determined. For comparison the spectra of fructose-6-phosphate and glucose-6-phosphate assays were also determined and are shown in Fig. 35. The glucose-6-phosphate assay was done with 3 mg. in a 2 ml. sample subjected to the fructose-6-phosphate estimation procedure. This represents a five fold excess over the maximum possible from the glucose-6-phosphate present in the digests as substrate for phosphoglucose isomerase, which therefore will provide little interferance in the assay of the digests for fructose-6-phosphate. In the case of the Cladophora extracts, which contained obvious activity, experiments were carried out to

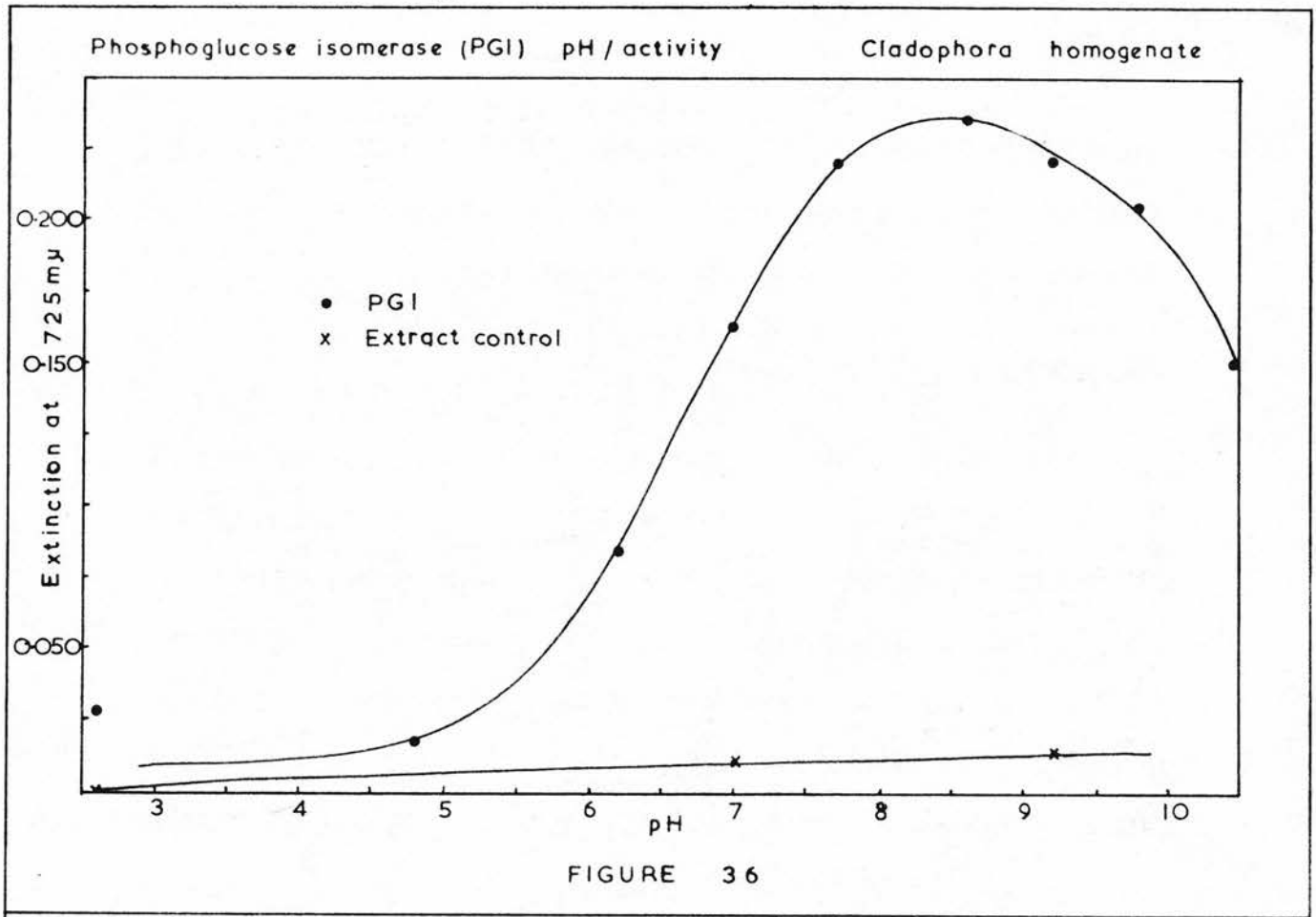


FIGURE 36

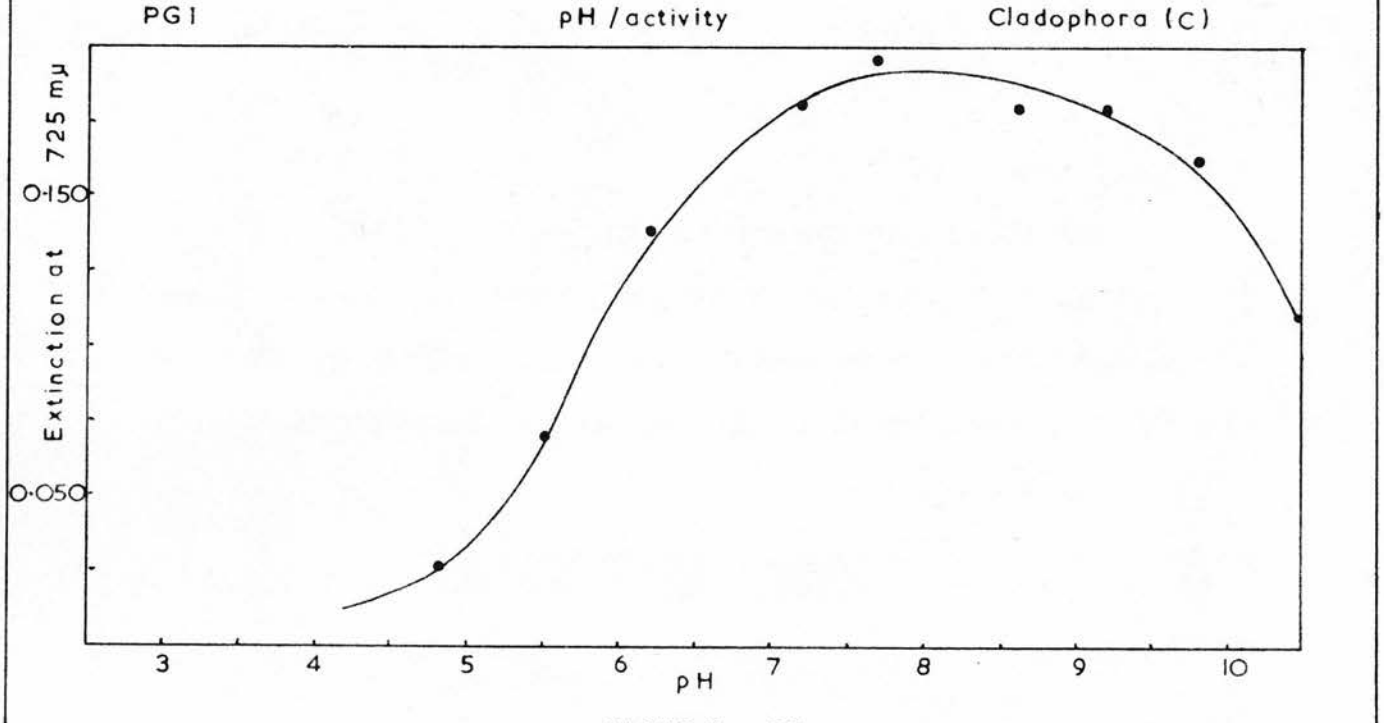


FIGURE 37

investigate the effect of pH and magnesium ions and to examine the ability of the extracts to effect the reverse reaction, converting fructose-6-phosphate to glucose-6-phosphate.

Cladophora

Standard digests were prepared with the Cladophora homogenate and incubated for 42 hr. The glucose-6-phosphate substrate solution contained 1.5 mg./ml. Extinction values obtained are quoted below. As mentioned in Methods and Materials, the development of the colour in this assay method is dependent on the conditions used. In this experiment the extinction values were very high, that of the pH 9.2 digest being 1.45. Therefore, extinction values were determined against that of the extract control at pH 2.6.

pH	2.6	4.8	6.2	7.0	7.7	8.6	9.2	9.8	10.5
	0.028	0.018	0.084	0.163	0.220	0.235	0.220	0.206	0.150
Extract controls	0			0.012			0.014		

These results are plotted in Fig. 36.

Similar digests were prepared with extract C solution (10 mg./ml.) with and without added magnesium chloride to a final concentration of 10 mM. After incubation for 20 hr. the following extinction results were obtained, read against water.

pH	4.8	5.5	6.2	7.2	7.7	8.6	9.2	9.8	10.5
without Mg	0.140	0.186	0.243	0.295	0.304	0.286	0.290	0.275	0.219
with Mg	0.135	0.175	0.253	0.288	0.308	0.292	0.289	0.273	0.222
Extract control					0.113 0.106				

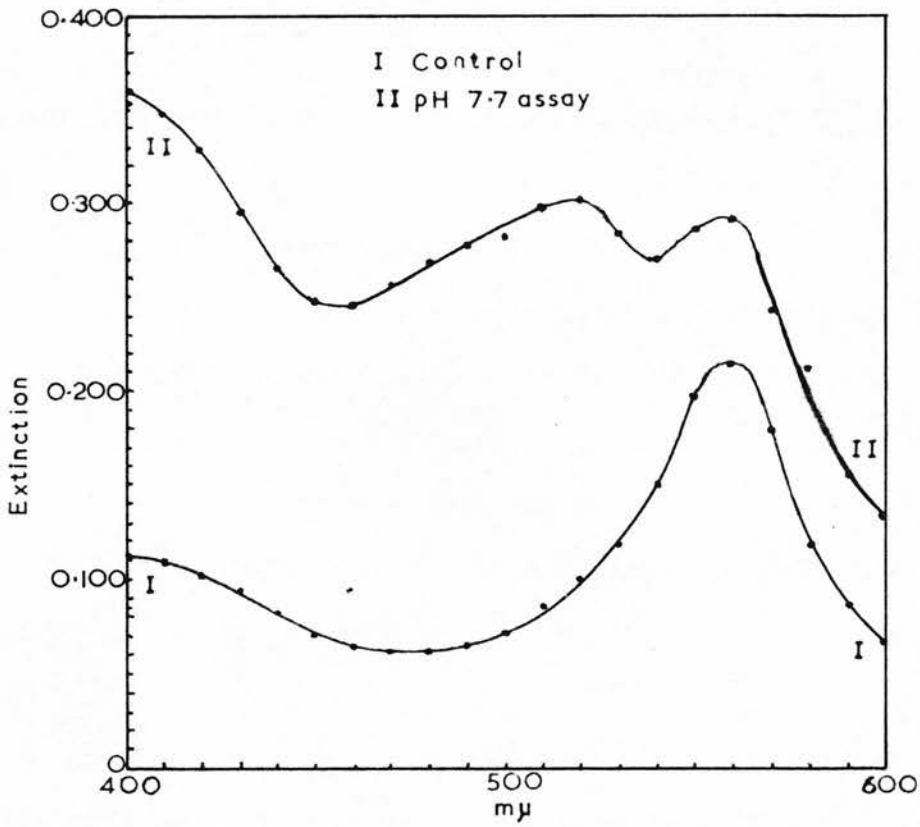


FIGURE 38

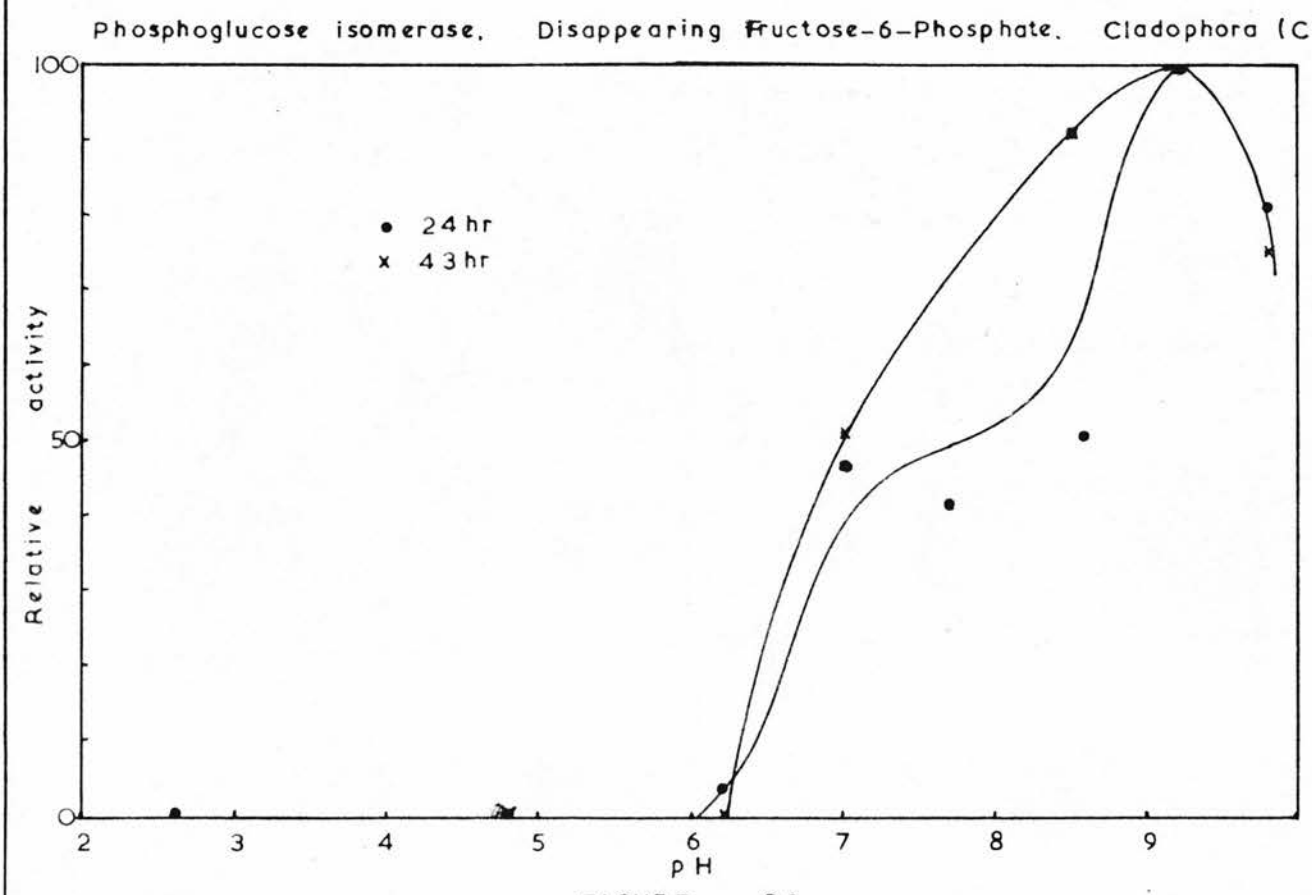


FIGURE 39

There is no significant difference between these two sets of results. The mean values are plotted in Fig. 37. Comparing this with Fig. 36 it is apparent that the phosphoglucose isomerase of Cladophora has a pH optimum range of about 7.5 - 9.2.

For comparison with the other results obtained below the absorption spectra of the extract control and of the pH 7.7 digest (without Mg) were determined and are shown in Fig. 38. It is obvious that the spectrum of the pH 7.7 digest is very similar to that of fructose-6-phosphate shown in Fig. 35 with that of the extract control superimposed.

Digests were prepared with a Cladophora homogenate and fructose-6-phosphate as substrate. The substrate solution was prepared from the barium salt by precipitating barium as barium sulphate with the calculated amount of potassium sulphate. The final concentration of fructose-6-phosphate (as free acid) was 0.7 mg./ml. This is equivalent to 1.1 mg./ml. as the barium salt. The controls noted in the results were also prepared. Samples (2 ml.) were taken after 24 and 43 hr. and assayed for residual fructose-6-phosphate. The extinction results obtained are given below.

24 hr.

pH	2.6	4.8	6.2	7.0	7.7	8.6	9.2	9.8
	0.833	0.835	0.829	0.775	0.783	0.771	0.710	0.734
Fructose-6-phosphate	0.252			0.253				0.256
Extract control				0.598				
Heated extract control				0.531				
Heated control + fructose-6-phosphate				0.822				

43 hr.

pH	4.8	6.2	7.0	8.6	9.2	9.8
	0.690	0.695	0.630	0.590	0.580	0.607
Fructose-6-phosphate			0.238			
Extract			0.482			
Heated extract			0.491			
Heated extract + fructose-6-phosphate			0.681			

In both cases the mean of the results for the pH 2.6 - 6.2 digests were taken as zero activity and the difference between these figures and those for the other digests used as a measure of the activity, expressed as values relative to a maximum of 100, in view of the variation in the absolute extinction results inherent in the assay method.

These figures are plotted in Fig. 39 and show optimum activity at pH 9.2. Considering the curves for the 24 and 43 hr. samples it is apparent that equilibrium between fructose-6-phosphate and glucose-6-phosphate is reached fastest at pH 9.2 with the digests at other pH values approaching the same equilibrium value. Comparing this

with the results from the glucose-6-phosphate digests the broad range of optimum activity (Figs. 36 and 37) suggests that equilibrium has been quickly established over this range of pH values. This is consistent with the phosphoglucose isomerase of Cladophora being of the normal type in which the equilibrium lies 70 - 30% in favour of glucose-6-phosphate (Slein, 1955).

Rhodymenia, Laminaria and Fucus

Standard digests were prepared with glucose-6-phosphate as substrate. Extract solutions used were Rhodymenia, both extracts 10 mg./ml., Laminaria, 20 mg./ml. and a Fucus homogenate. The activity was examined in digests at pH 4.8, 7.0 and 9.2 with an extract control at pH 7.0. Samples were taken after 40-50 hr. incubation and subjected to the fructose-6-phosphate assay procedure. Extinctions were determined at 520 and 560 mμ and absorption spectra were obtained as indicated.

Rhodymenia

Extract P 40 hr.

pH	4.8	7.0	9.2	extract control
E at 520 mμ	0.720	0.106	0.076	0.092
560 mμ	0.126	0.206	0.130	0.205
E_{520}/E_{560}	0.57	0.52	0.58	0.45

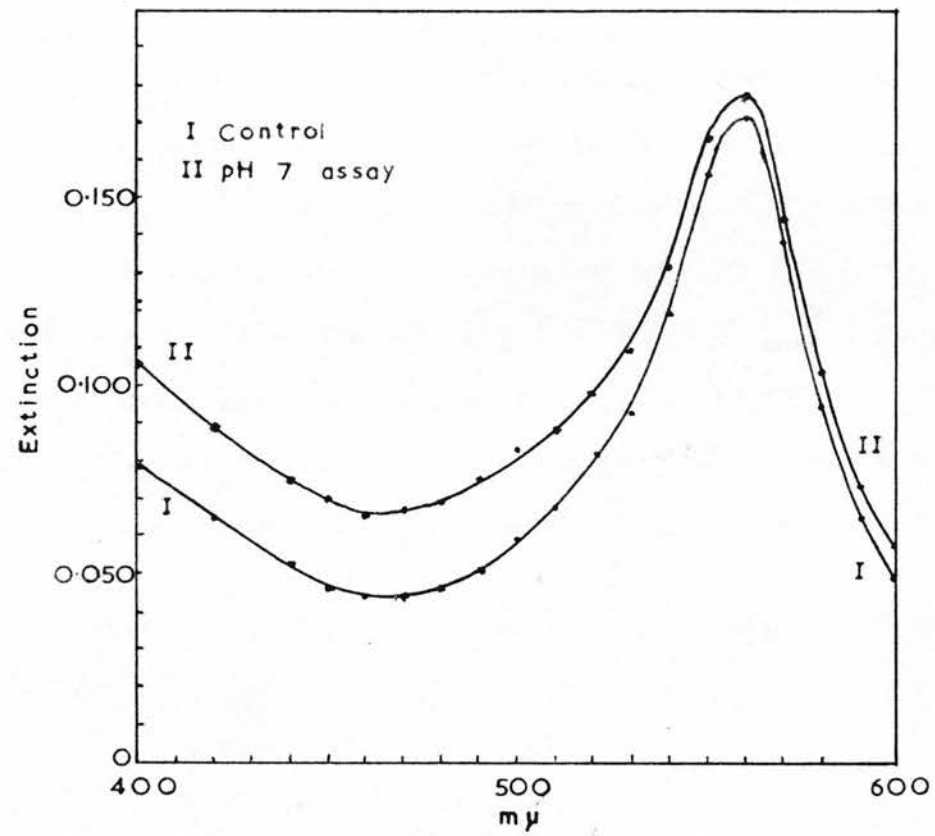


FIGURE 40

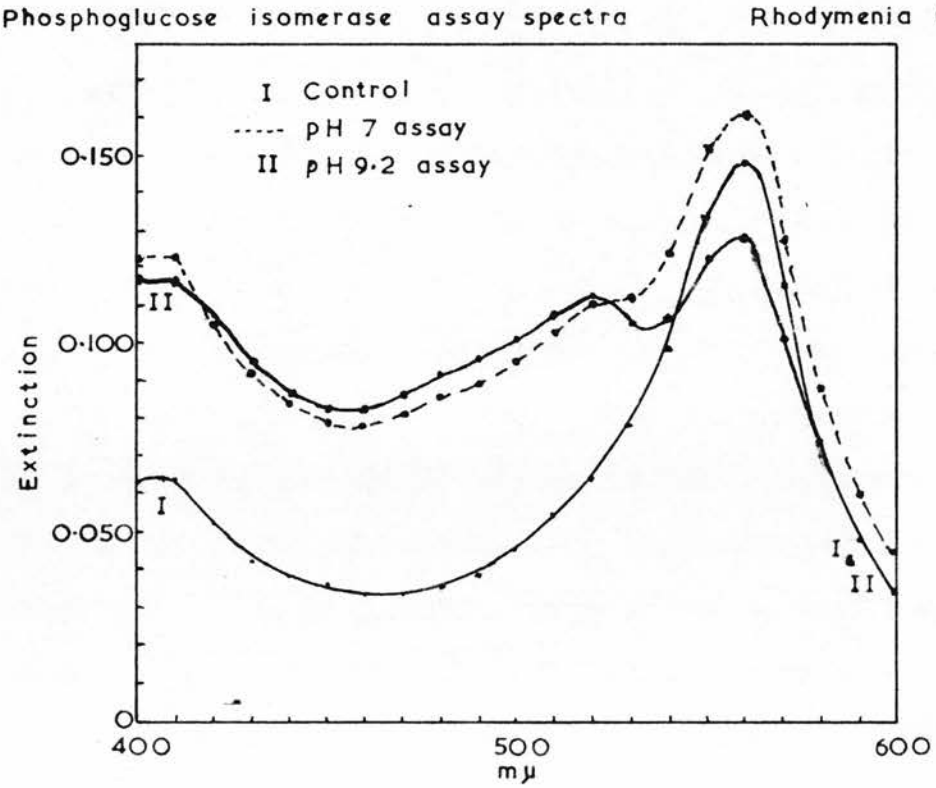


FIGURE 41

The absorption spectra of the pH 7.0 digest assay and that of the extract control were obtained and are shown in Fig. 40. No evidence of a peak at 520 mμ was obtained in the spectrum of the pH 7.0 digest assay and therefore no phosphoglucose isomerase activity is present in extract P of Rhodymenia. The slight variation in the ratios of extinction values between the digests containing substrate and the extract control was probably due to a small contribution to the E_{520} figure by the glucose-6-phosphate. (See glucose-6-phosphate spectrum, Fig. 35).

Extract C 30 hr.

pH	4.8	7.0	9.2	extract control
E_{520}	0.074	0.111	0.120	0.077
E_{560}	0.138	0.190	0.166	0.179
E_{520}/E_{560}	0.53	0.59	0.72	0.43

The absorption spectra of digests 7.0 and 9.2 and of the extract control were determined after 40 hr. incubation and are shown in Fig. 41. The ratios of extinction values at 520 and 560 mμ were 0.68, 0.87 and 0.42 respectively. These results, together with the evidence from the spectra, show that this extract of Rhodymenia contains weak phosphoglucose isomerase activity, with greatest activity at pH 9.2, indicated by the appearance of a distinct peak at 520 mμ characteristic of fructose-6-phosphate. The presence of weaker activity in the pH 7.0 digest is shown by the marked variation of its spectrum from that of the extract control with a slight inflection at 520 mμ and by the substantial variation in the ratio of the extinction values at 520 and 560 mμ, from that of the extract control.

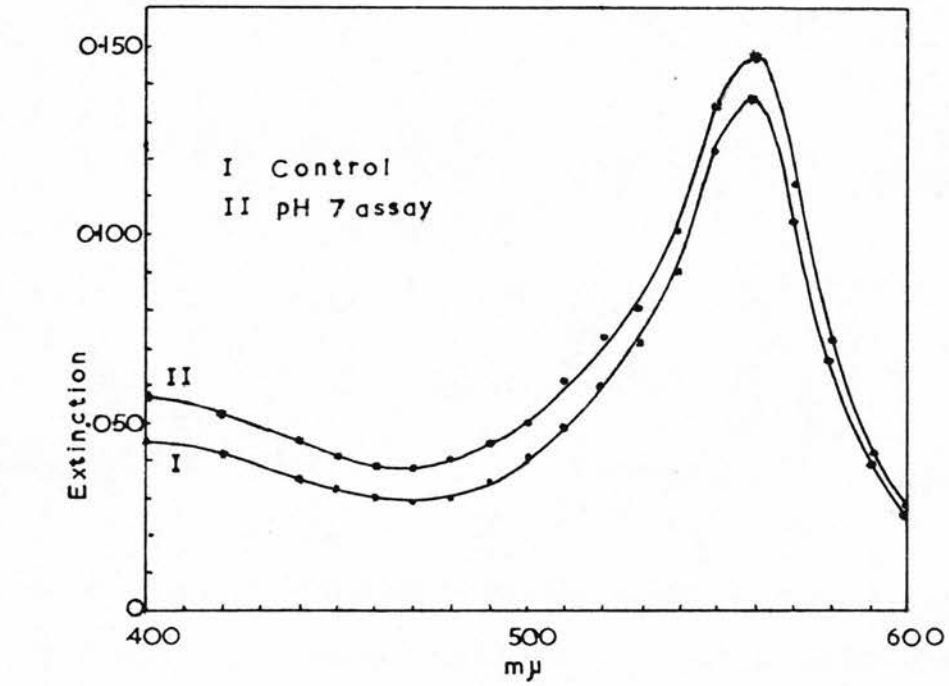


FIGURE 42

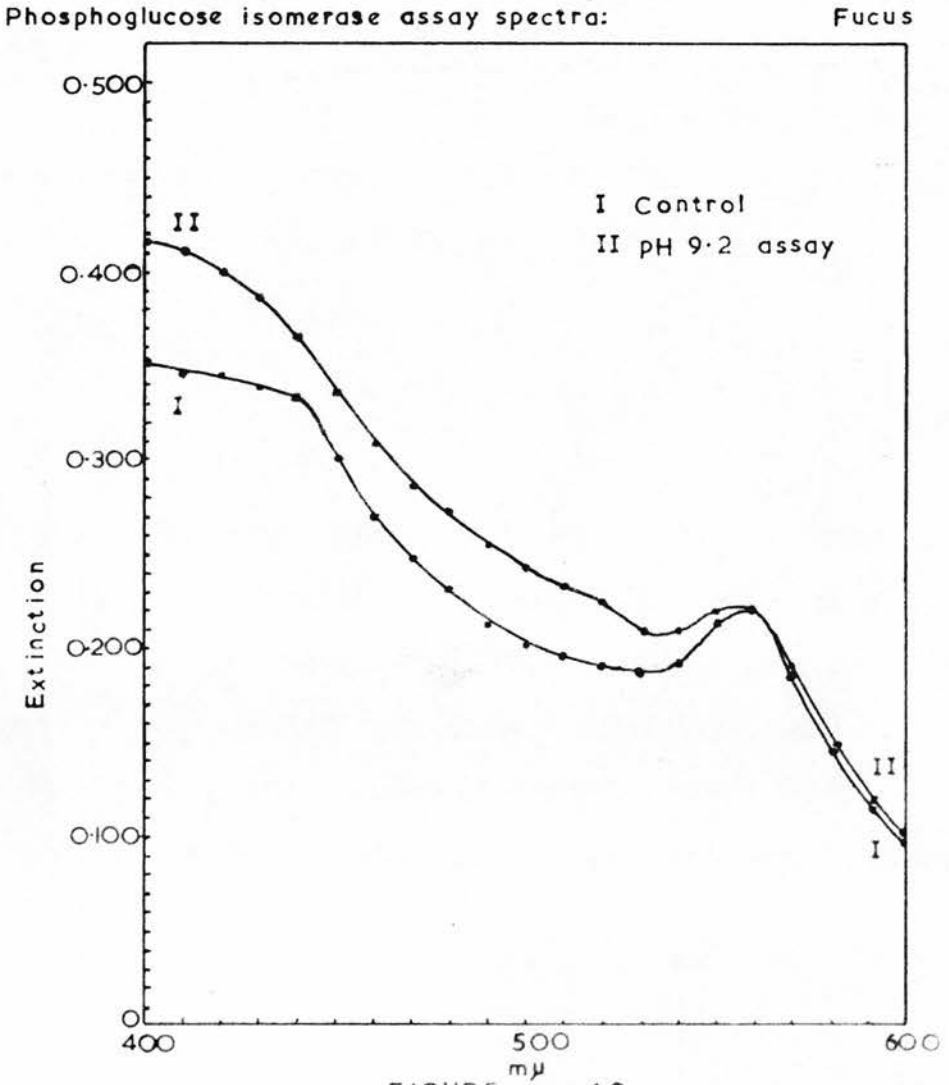


FIGURE 43

Laminaria

pH	4.8	7.0	9.2	extract control
E_{520}	0.064	0.081	0.064	0.060
E_{560}	0.127	0.164	0.117	0.145
E_{520}/E_{560}	0.50	0.50	0.55	0.42

The spectra of the pH 7.0 and extract control assays were determined and are shown in Fig. 42. These give no evidence of phosphoglucose isomerase in the Laminaria extract, the two curves differing only by an amount attributable to the added glucose-6-phosphate.

Fucus

pH	4.8	7.0	9.2	extract control
E_{520}	0.092	0.137	0.155	0.119
E_{560}	0.109	0.137	0.139	0.128
E_{520}/E_{560}	0.86	1.0	1.12	0.93

Absorption spectra of the pH 9.2 digest assay and that of the extract control were determined and are shown in Fig. 43. Again no evidence of phosphoglucose isomerase was obtained. The increasing E_{520} with increasing pH was shown by the following series of extract controls to be due to the brown colouration developed in digests containing Fucus extracts. (cf. Phosphomonoesterase section above).

pH	4.8	7.0	9.2
E_{520}	0.115	0.156	0.172

In view of this demonstration of the absence of phosphoglucose isomerase activity in a fresh algal extract, (cf. marked activity of the Cladophora homogenate) the effect of the Fucus homogenate on the phosphoglucose isomerase activity of almond emulsin was investigated in digests at pH 8.6. Three digests were prepared, two standard digests with Fucus homogenate and emulsin solution (1 mg./ml.) as the extract solutions and a third containing 1 ml. of each extract solution, to the exclusion of 1 ml. of water. Extract controls were also prepared. After 18 hr. incubation samples were taken and assayed for fructose-6-phosphate. Extinction results are quoted below.

Fucus 0.169; Emulsin 0.363; Fucus + Emulsin 0.169.

Fucus control 0.154; Emulsin control 0.081.

The Fucus extract therefore inhibits the phosphoglucose isomerase activity of the emulsin, making any investigation of this activity in crude extracts of Fucus impossible.

Phosphoglucomutase activity

The various algal extracts were examined for phosphoglucomutase in standard digests with glucose-1-phosphate (1 mg./ml. substrate solution) as substrate. The glucose-1-phosphate used, which was kindly provided by Dr. W.J. Whelan, had been prepared enzymically and thus was believed to contain glucose 1, 6 diphosphate, a necessary co-factor for phosphoglucomutase. Magnesium chloride to a final concentration of 10 mM was also added to the digests as magnesium ions too are required for optimum activity (Najjar, 1955). Extracts were examined in

single digests at pH 7.7, this being within the range 7.6 - 9.2 quoted by Cori et al (1938b) for muscle enzyme and close to the figure of 7.5 quoted by Cardini (1951) for the enzyme from Jack beans. The digests were examined for possible activity by the method described by Cori et al (1938b) utilising the relative stabilities of the glucose-1 and -6-phosphates, the former being completely hydrolysed to glucose and phosphoric acid by treatment with N sulphuric acid at 98°C. for 5 min. while the latter is stable under these conditions. Digest samples (1 ml.) were added to 2 N sulphuric acid (1 ml.) and heated on a boiling waterbath for 5 min. After cooling, these solutions were assayed for inorganic phosphorus in the usual way. Any decrease in the inorganic phosphorus determined by this method, relative to the sum of a glucose-1-phosphate and an extract control would represent some conversion of the 1-phosphate into the stable 6-phosphate by phosphoglucomutase.

Extract solutions used were, Cladophora extract C (10 mg./ml.) Rhodymenia extracts P and C (10 mg./ml.), Laminaria (20 mg./ml.) and a Fucus homogenate. Controls on the method were also prepared using Jack bean flour (20 mg./ml.) and almond emulsin (5 mg./ml.). The first of these was to check that under the conditions used, the assay conditions were satisfactory, and the second, to show that the glucose-1-phosphate did contain sufficient glucose 1, 6 diphosphate as co-factor for any phosphoglucomutase activity present to be apparent, as the Jack bean preparation could contain sufficient of this compound to exhibit phosphoglucomutase without the necessary co-factor being present in the glucose-1-phosphate preparation.

The extracts were surveyed in three separate groups of digests. After incubation for the stated time digest samples (2 ml. and 1 ml.) were assayed for phosphatase and phosphoglucomutase activity. In order that a direct comparison may be made, the extinction results quoted below for the phosphoglucomutase assay have been corrected for a 2 ml. sample by doubling the experimental results.

1. Incubation period 25 hr.

Digest	Phosphatase	Phosphoglucomutase
Cladophora	0.336	0.846
Jack bean	0.403	0.448
Emulsin	0.032	0.530
G-1-P control	-	0.874
Extract controls		
Cladophora	0.064	0.066
Jack bean	0.044	0.046
Emulsin	0.013	0.014

The Jack bean and emulsin controls show that the assay method is valid, there being evidence of phosphoglucomutase activity in both digests. The substantial difference between the Cladophora digest result and the sum of glucose-1-phosphate and Cladophora extract controls indicates the presence of weak phosphoglucomutase in the Cladophora extract but the further investigation of this activity in the presence of a relatively more active phosphatase was impracticable.

2. Incubation period 40 hr.

Digest	Phosphatase	Phosphoglucomutase
Rhodymenia (P)	0.157	0.984
Fucus	0.542	1.30
Extract controls		
Rhodymenia (P)	0.105	0.098
Fucus	0.435	0.440
G-1-P	-	0.872

There is no evidence of any phosphoglucomutase activity present in these extracts.

3. Incubation period 46 hr.

Digest	Phosphatase	Phosphoglucomutase
Rhodymenia (C)	0.142	0.920
Laminaria	0.057	0.856
Extract controls		
Rhodymenia (C)	0.067	0.068
Laminaria	0.009	0.012
G-1-P	-	0.844

Again there is no evidence of any phosphoglucomutase activity in these extracts.

Thus, only the Cladophora extract gave any evidence of phosphoglucomutase activity, and that only very weak.

Discussion

In the experiments described in this chapter the algal extracts have been shown to contain a variety of phosphomonoesterase activities. The major activities are outlined in the table below:-

Extract		Incubation time (hr.)	Percentage hydrolysis				
			pH 4.8	7.0	8.6	9.2	10.5
Cladophora							
Homogenate (Fig. 23)		20					50
Extract C	No Mg	20		11			17
	Mg (Fig. 25)	20		14			30
Rhodymenia							
Extract P	No Mg	42	18		10		
	Mg (Fig. 26)	42	12		14		
Extract C	No Mg	42	10		9		7
	Mg (Fig. 27)	42	6		14		14
Laminaria							
	No Mg	43		5.5		6	
	Mg (Fig. 28)	43		4.5		3.5	
Fucus							
	No Mg	42		5			10
	Mg (Fig. 29)	42		10			16

The only major acid phosphomonoesterase activity is that in the Rhodymenia extracts. The pH optimum and the inhibitory effect of added magnesium ions indicate that this is a phosphomonoesterase type III. Some evidence of a similar activity in the Cladophora homogenate (Fig. 23) is available in that β-glycerophosphate is hydrolysed by this extract between pH 2.6 and 4.8 whereas no hydrolysis occurs in this range using the Cladophora extract C. Some slight inhibition of the activity of this extract at pH 4.8 and 5.5 also indicates the presence of weak phosphomonoesterase type III (Fig. 25).

Extracts of both brown algae show distinct activities with optimum pH of about 7. Both Rhodymenia extracts too, show apparent optima at about this pH but, as previously mentioned these may be due to a summation of declining and increasing activities on either side. The Cladophora extracts show activity in the pH range 6.2 - 7.7 but no distinct optimum is apparent.

Both Rhodymenia extracts and the Laminaria extract contain alkaline phosphatase activity with optimum activity in the range 8.6 - 9.2. The Rhodymenia activity is of the phosphomonoesterase type I being activated by magnesium ions. The Laminaria activity is very weak and as previously mentioned little significance may be attached to the apparent inhibitory effect of added magnesium ions. In Cladophora extracts no evidence of marked type I activity was obtained. Considering both the pH/activity curves obtained with Fucus extracts with β-glycerophosphate and mannitol-1-phosphate as substrate (Figs. 29 and 33) there is some indication of phosphomonoesterase type I activity but it is weaker than what appears to be the peculiarly algal alkaline

phosphomonoesterase with high optimum pH which may mask any type I activity.

This activity with optimum pH of 10.5 or higher has been demonstrated in extracts of Cladophora, Rhodymenia and Fucus. This was the most active phosphatase in the Cladophora homogenate which caused approximately five times more hydrolysis at pH 10.5 than at pH 7.0. This activity was destroyed by heat treatment and was therefore assumed to be due to enzymic hydrolysis. The activity is increased by 50-100% in the various extracts in the presence of 10 mM magnesium. Using Cladophora homogenate in experiments to investigate glucose-6-phosphatase activity it was found that on prolonged incubation the activity at pH 9.8 became greater than that at pH 10.5 indicating that the true optimum pH is at pH 9.8, the enzyme being progressively denatured at pH 10.5.

In a preliminary survey, only Cladophora extracts showed any marked hydrolytic activity towards glucose-6-phosphate. Examination of this activity has shown that glucose-6-phosphate is a better substrate for this algal enzyme than β-glycerophosphate in the pH range 6.2 - 7.7. Using a homogenate as enzyme source the ratio of activity at pH 7.7 to that at pH 9.8 with β-glycerophosphate as substrate was 0.25 and with glucose-6-phosphate as substrate 0.50. Using extract C the respective ratios were 0.67 and 1.3 in digests containing no magnesium. Thus by changing the substrate an approximately two-fold increase in activity at pH 7.7 relative to that at pH 9.8 has been achieved. In view of the substantial hydrolysis of β-glycerophosphate in the pH range 6.2 - 7.7 it is unlikely that this activity is due to a specific glucose-6-phosphatase.

In an attempt to demonstrate part of the pathway of mannitol synthesis from fructose-6-phosphate via mannitol-1-phosphate outlined in the introduction to this chapter, the hydrolytic activity of the two

brown seaweed extracts towards mannitol-1-phosphate was examined. No evidence of any specific mannitol-1-phosphatase was obtained, the substrate being hydrolysed in essentially the same pattern as sodium β-glycerophosphate.

Phosphoglucose isomerase has been demonstrated in the Cladophora extracts. Variation in the rates of achieving equilibrium with glucose-6-phosphate and with fructose-6-phosphate as substrate suggest that the equilibrium mixture is similar to that from other sources i.e. 70% glucose-6-phosphate and 30% fructose-6-phosphate (Slein, 1955). In digests with fructose-6-phosphate as substrate approximate equilibrium is reached first at pH 9.2, indicating optimum activity at this pH while with glucose-6-phosphate as substrate approximate equilibrium is quickly reached over the range 7.0 - 9.0. Ramasarma and Giri (1956) reported a similar variation in pH optima quoting pH 7.8 - 8.0 with glucose-6-phosphate as substrate and 9.0 with fructose-6-phosphate as substrate, using extracts of Phaseolus radiatus. Noltmann and Bruns (1959) and Singh (1959) report pH optima of 7.8 - 8.0 for the enzymes from yeast and Aspergillus niger respectively. Slein (1955) reported pH 9.0 as the optimum pH of the muscle enzyme.

Weak phosphoglucose isomerase has been demonstrated in Rhodymenia extract C with activity greater at pH 9.2 than at pH 7.0. This agrees with the pH optima figures quoted above. It is of interest to note that no activity could be shown in extract P. The weak activity was probably destroyed during the extraction since this was effected with phosphate/citric acid buffer and Slein (1955) reported that phosphate ions inhibited the phosphoglucose isomerase from muscle.

Since the phosphoglucose isomerase of almond emulsin was completely inhibited by the Fucus homogenate the demonstration of this activity in crude Fucus extracts is impossible. No evidence of phosphoglucose isomerase activity was found in the Laminaria extract.

Of all the algal extracts examined only Cladophora extract C showed very weak phosphoglucomutase activity.

Considering the enzymic activity demonstrated by the experiments described in this chapter the Cladophora extracts appear to contain a higher level of activity than the Rhodymenia extracts. Comparing the reverse properties of the extracts with respect to the β-glycosidase activity examined in the previous chapter, this variation in activity cannot be wholly attributed to inherent weak activity in the extracts and therefore it may be related to the metabolic significance of these activities in the hexose metabolism of the two algae.

As proposed by Jacobi (1957a and b), the green algae, here typified by Cladophora rupestris, have a hexose metabolic pattern similar to that of higher plants and animals, but with some marked differences. He demonstrated fructose diphosphate aldolase and glucose-6-phosphate dehydrogenase in extracts of green algae but was unable to detect hexokinase or phosphoglucomutase. In the present work extracts of Cladophora have been shown to contain phosphoglucose isomerase and phosphoglucomutase, both enzymes taking part in the hexose metabolism of higher plants and animals (Fig. 3). In view of the reported presence of a starch-type polysaccharide in Cladophora (Love et al 1963) the very weak phosphoglucomutase activity indicates that the synthesis of starch by phosphorylase acting on glucose-1-phosphate is not a major pathway to

this polysaccharide. This is in agreement with the failure of Duncan (1956) to demonstrate any phosphorylase activity in Cladophora extracts.

On the other hand Jacobi (1957a and b) showed that the hexose metabolism of the red algae must be substantially different from that in higher plants and animals, being unable to detect any of the enzymes of the glycolytic cycle in a number of red algae. The very weak phosphoglucose isomerase demonstrated in the Rhodymenia extract during this present work confirms this suggestion but indicates that the interconversion of glucose-6-phosphate and fructose-6-phosphate has some significance in the hexose metabolism of the alga.

The enzymic activity of the Laminaria extract used was generally very weak, (cf. β-glycosidase activity examined in the previous chapter) and therefore little significance may be attached to the absence of phosphoglucose isomerase and phosphoglucomutase in this extract. The inhibitory effect of Fucus extracts on established phosphoglucose isomerase activity in the almond emulsin made the detection of this activity impossible in the crude extracts and raises the question of the ability of the seaweed to keep any possible enzyme and the potential inhibitor apart.

Summarising the results obtained in this chapter, the algal extracts have been shown to contain essentially similar phosphomonoesterase activity to other plant and animal sources but with no apparent bias towards either acid or alkaline activities. The exception to this similarity is the presence of a magnesium activated, alkaline phosphomonoesterase with the unusually high pH optimum of 9.8 shown to occur in extracts of green, red and brown algae.

Cladophora extracts have been shown to hydrolyse glucose-6-phosphate more readily than β-glycerophosphate in the pH range 6.2 - 7.7 but no evidence of specific mannitol-1-phosphatase was found in either of the brown seaweed extracts.

Phosphoglucose isomerase and very weak phosphoglucomutase has been detected in Cladophora extracts. Weak phosphoglucose isomerase has been demonstrated in an extract of Rhodymenia. The significance of these activities in relation to the hexose metabolism of these two algae has been discussed.

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APPENDIX

The following abstract of a paper to be read at the 431st Meeting of the Biochemical Society will be published in the Biochemical Journal.

The Fine-structure of Rhodymenia palmata Xylan

by D.J. Manners and J.P. Mitchell (Department of Chemistry, University of Edinburgh)

Previous workers (Percival & Chanda, 1950; Barry, Dillon, Hawkins & O'Colla, 1950) have shown that Rhodymenia palmata xylan is a polymer of β-D-xylopyranose residues containing about 80% of (1→4) and 20% of (1→3) linkages. The average chain length of one sample, by methylation analysis, was 20-21 and the apparent degree of polymerisation of a second sample, by periodate oxidation analysis, was 39-40. We now report the results of further structural studies.

The proportion of triol-groups in certain polysaccharides may be determined from the production of formic acid following periodate oxidation at 2°C. under conditions in which hydrolysis of intermediary formyl ester and 'over-oxidation' does not occur. Application of this method to two samples of xylan, one prepared by acid-extraction of Rhodymenia palmata, and the other (kindly provided by Dr. R.A. Wall) by extraction with butanol-water mixtures, gave one triol-group per 31 and 65 xylose residues respectively. These results, assuming a linear structure, correspond to minimum degrees of polymerisation of 54 and 114, suggesting that the xylan is a rather larger molecule than hitherto believed, and that most samples previously examined may have been inadvertantly degraded during acid-extraction.

Application of the reaction series periodate oxidation, reduction with borohydride, and partial acid hydrolysis (cf. Smith & Montgomery, 1959) to Rhodymenia palmata xylan gave a complex mixture of products including xylose, xylitol, glycerol and milligram quantities of

xylosyl-glycerol, rhodymenabiose O-β-D-xylopyranosyl-(1→3)-D-xylopyranose and a derivative of this disaccharide. The latter two products were characterised by chromatographic and electrophoretic properties and and micro-scale methylation analysis. Their presence indicates that a small proportion of adjacent (1→3)-linked xylose residues are located in the xylan chain although the major proportion of (1→3)-linked xylose residues are flanked by (1→4)-linked residues.

These conclusions were confirmed by degradation of the xylan with an enzyme preparation from Myrothecium verrucaria (cf. Bishop & Whitaker, 1955). The products included xylose, xylobiose, xylotriose, xylotetraose, small quantities of rhodymenabiose and 3^2-β-xylosyl xylobiose and trace amounts of oligosaccharides tentatively identified as 4^2-β-xylosyl rhodymenabiose and rhodymenatriose. Control experiments showed that the enzyme preparation did not produce oligosaccharides containing β-(1→3)-xylosidic linkages by trans-β-xylosylation from xylobiose.

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